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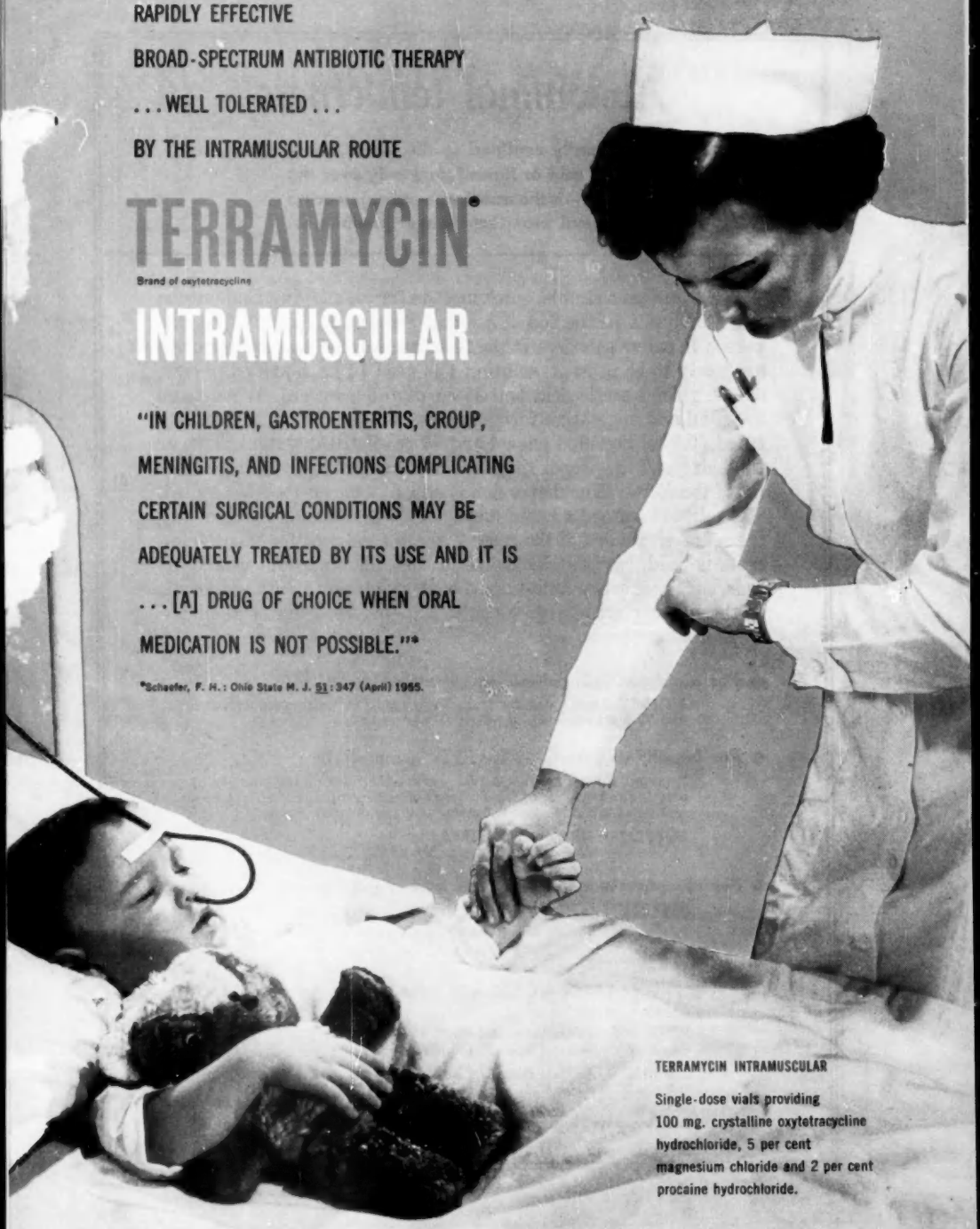

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1. Martin, G. A., Garfinkel, B. T., Brooke, M. M., Weinstein, P. P., and Frye, W. W.: *J.A.M.A.*, 151:1055, Mar. 28, 1953.
2. Kohn, J.: *Jour. Trop. Med.*, 53:212, Nov., 1950.
3. Information Please: *GP*, 4:91, Sept., 1951.

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CONTENTS

Relapses with Chesson strain *Plasmodium vivax* following treatment with chloroquine. GEOFFREY M. JEFFERY 1
Further studies on cross-resistance between pyrimethamine and related compounds. JOSEPH GREENBERG AND HOWARD W. BOND 14
Differences in the course of *Plasmodium berghei* infections in some hybrid and backcross mice. JOSEPH GREENBERG 19
Intestinal parasitic infections in Forsyth County, North Carolina. IV. Domestic environmental sanitation and the prevalence of *Entamoeba histolytica*. T. T. MACKIE, J. W. MACKIE, C. M. VAUGHN, N. N. GLEASON, B. G. GREENBERG, E. S. NENNINGER, M. N. LUNDE, L. L. A. MOORE, J. A. KLUTTZ AND M. O. TALIAFERO 29
Intestinal parasitic infections in Forsyth County, North Carolina. V. Prevalences of individual parasites. T. T. MACKIE, J. W. MACKIE, C. M. VAUGHN, N. N. GLEASON, B. G. GREENBERG, E. S. NENNINGER, M. N. LUNDE, L. L. A. MOORE, J. A. KLUTTZ AND M. O. TALIAFERO 49
The effect of rabbit antisera on the ability of *Entamoeba histolytica* to phagocytose red blood cells. JAMES G. SHAFFER AND JOSEPH ANSFIELD 53
Glucosamine in the culture of *Entamoeba histolytica* with a mixed bacterial flora. JOSEPH GREENBERG, D. JANE TAYLOR AND HOWARD W. BOND 62
The use of fumagillin in the treatment of amebiasis. GAMAL NOR EL-DIN 67
Erythromycin in the treatment of amebiasis. GAMEL NOR EL-DIN 72
West Nile virus infection in arthropods. HERBERT S. HURLBUT 76
The transmission of Western equine encephalitis virus by the mosquito *Culex tarsalis* Coq. HERBERT C. BARNETT 86
Experimental studies of concurrent infection of canaries and of the mosquito *Culex tarsalis* with *Plasmodium relictum* and Western equine encephalitis virus. HERBERT C. BARNETT 99
Histoplasmin sensitivity in Indonesia. LIE KIAN JOE, NJO-INJO TJOEI ENG, PHYLLIS Q. EDWARDS AND FREDRIK DECK 110
Studies in shigellosis. V. The relationship of age to the incidence of *Shigella* infections in Egyptian children, with special reference to shigellosis in the newborn and in infants in the first six months of life. T. M. FLOYD, A. R. HIGGINS AND M. A. KADER 119
A method for the isolation of *Salmonella typhi* from blood clot. KENNETH C. WATSON 131
The dried filter paper technique for sending stool specimens to a laboratory for bacteriologic examination. LIE KIAN JOE 133
On the infectivity of the Formosan strain of *Schistosoma japonicum* in macaques. H. F. HSÜ AND S. Y. LI HSÜ 136
Schistosomiasis in Saudi Arabia. Treatment with lucanthone hydrochloride (Nilodin) and with sodium antimonyl gluconate (Triostam). M. L. TARIZZO 145
Fatal hematemesis in *Schistosoma mansoni* infection. BEN C. WILLARD, JR. 150
Oral and intraperitoneal treatment of *Acanthocheilonema* (*Dipetalonema*) *perstans* with diethylcarbamazine. H. STROHSCHNEIDER 158
Effect of topically applied DDT on development of *Plasmodium vivax* and *P. falciparum* in *Anopheles quadrimaculatus*. ROBERT W. BURGESS 163
Infectivity of *Plasmodium berghei* for *Anopheles quadrimaculatus* and other mosquitoes. BETTIE L. CELAYA, EDITH D. BOX, AND WENDELL D. GINGRICH 168
The loss and redevelopment of insecticide resistance in Egyptian house flies. ROBERT L. PEFFLY AND A. A. SHAWARBY 183
Efficacy of the clothing impregnants M-2065 and M-2066 against terrestrial leeches in North Borneo. BRYCE C. WALTON, ROBERT TRAUB AND HAROLD D. NEWSON 190
Books received 196

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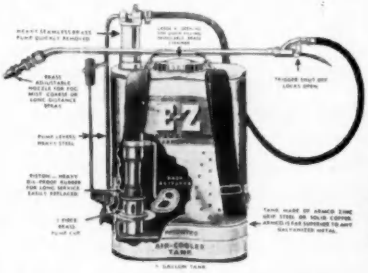

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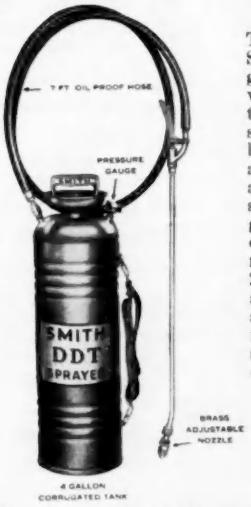

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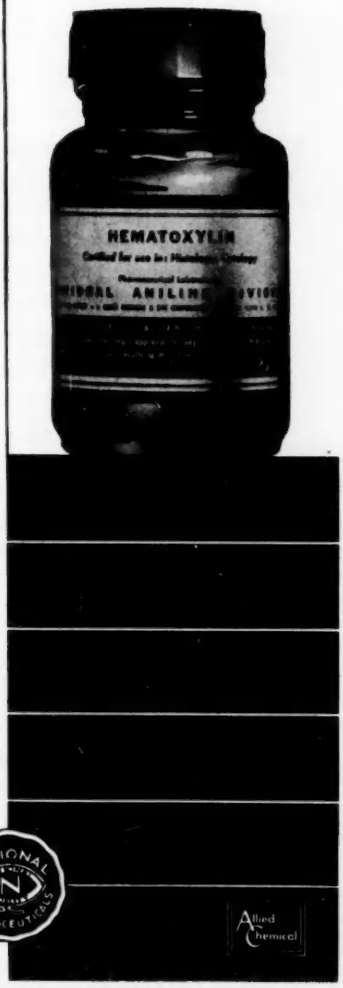

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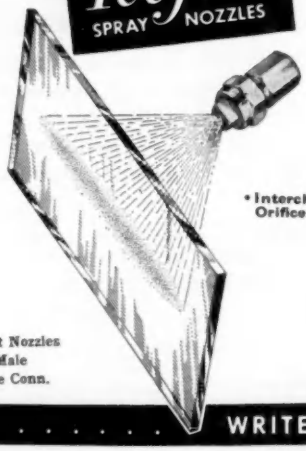

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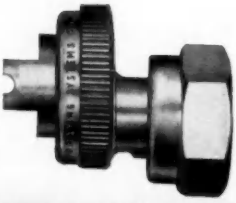

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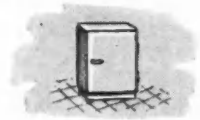

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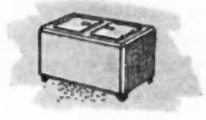

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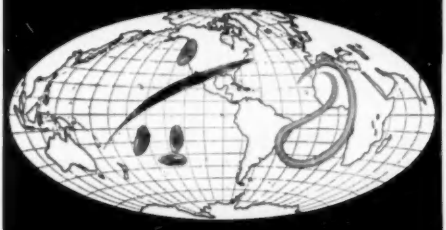

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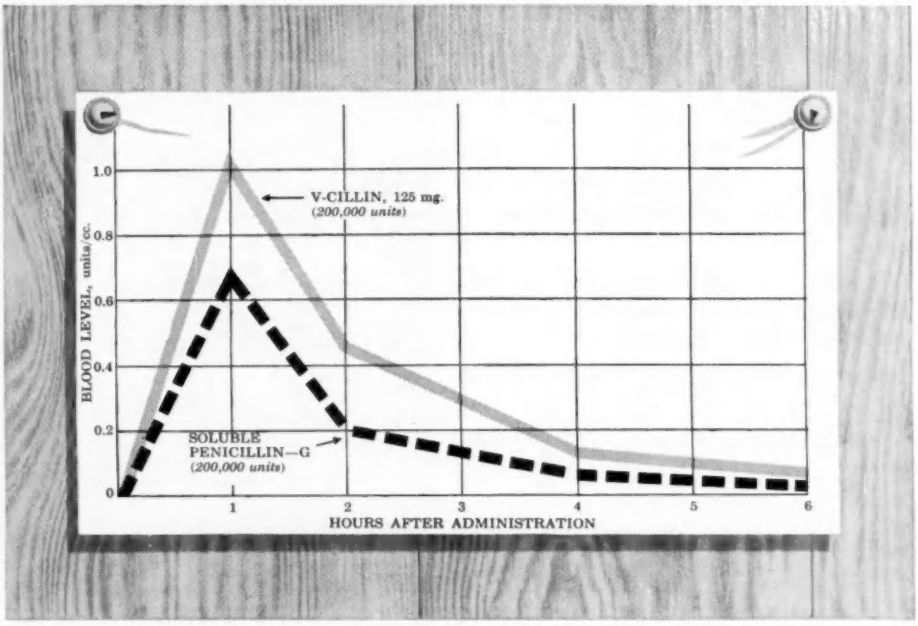

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RELAPSES WITH CHESSON STRAIN *PLASMODIUM VIVAX* FOLLOWING TREATMENT WITH CHLOROQUINE

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There are several reports in the literature concerning the relapse rate of experimental Chesson strain *Plasmodium vivax* following treatment with various dosages of chloroquine, but in all cases this work was done in volunteers in whom the infection was necessarily terminated prior to any extensive clinical attack. Although this type of treatment subjected the drug to a most severe challenge, in that its action was not aided to any great extent by developed immunity in the patient, it still does not simulate that which would be administered to many naturally acquired primary cases of *vivax* malaria in an endemic situation. Here treatment often would be after a number of days of clinical malaria and it is presumed that this would alter the response to the drug considerably.

The use of Chesson *P. vivax* in the malaria therapy of neurosyphilis has given the opportunity to observe the relapse rate and pattern after treatment with chloroquine of a number of sporozoite-induced clinical infections. The results of these studies may clarify the effect of the drug on infections acquired and treated in certain endemic areas rather than under less natural experimental conditions.

METHODS

The patients studied were white neurosyphilitics, with a few exceptions to be noted later. The *P. vivax* (Chesson) infection was induced by allowing infected *Anopheles quadrimaculatus* to feed on the patient. The number of infective bites was determined by postfeeding dissection of the specimens and examination for sporozoites in the salivary glands. The number of infective bites ranged from 3 to 100 with a median of 10; in more than half of the cases 7 to 14 infective bites were administered.

Blood smears of the Earle-Perez type were started six days after inoculation and were continued until the blood was negative for three consecutive days following treatment. Parasitemias were expressed in numbers per cmm. After becoming negative for parasites the patients were followed twice weekly with ordinary thick-thin blood films. In the event of recurrent parasitemia, Earle-Perez smears were again started. Patients were followed for as long as possible after last parasite presence, usually for a period of one year. In most cases the total observation period was in excess of one year, and in many cases for two, three, or even four years.

Chloroquine was given in two forms. The routine oral treatment was as the diphosphate in tablets containing 0.3 gram of base. A total of 1.5 grams of base was given over a period of three days. On the first day of drug two tablets (0.6

TABLE 1

Treatment of P. vivax *(Chesson) primary attacks with 1.5 grams chloroquine base orally*

Patient no.	No. of infective mosq. bites	Day of parasite patency	Previous no. of paroxysms	Parasites per cmm. at R_x	Last parasite day*	Last day fever 100°*	Relapse: days after treatment‡
G 52	7	29	20	37,440	D + 3	?	95
G 55	9	17	16	32,160	D + 5	D + 2	49
G 65	10	27	26	22,680	D + 4	D + 1	102
G 66	9	24	22	14,160	D + 1	D	95
G 78	15	9	9	18,360	D + 1	D + 1	93
G 82	24	9	6	31,680	D + 3	D + 2	149
G 86	4	19	16	18,000	D + 3	D	98
G 111	3	28	13	12,060	D + 1	D	?
G 112	12	12	9	22,200	D + 2	D + 1	43
G 126	10	8	8	29,280	D + 2	D + 3	78
G 135	11	15	10	540	D + 1	D − 2	60
G 175	3	6	3	5,320	D + 2	D + 1	123
G 192	24	26	17	27,120	D + 2	D	?
G 196	14	22	22	14,240	D + 2	D	73
G 213	34	11	8	32,640	D + 3	D + 2	105
G 214	16	10	9	10,800	D + 3	D + 1	?
G 215	43	10	7	24,720	D + 2	D − 2	112
G 216	45	11	7	82,800	D + 4	D + 1	106
G 223	8	19	8	16,072	D + 2	D	50
G 244	11	11	10	810	D + 1	D − 2	112
G 249	15	18	13	6,840	D + 5	D + 1	?
G 252	6	31	23	5,376	D + 1	D	103
G 266	50	71	32	2,040	D + 1	D − 14	322
G 267	50	8	7	3,000	D + 4	D − 2	59
G 276	4	27	16	12,720	D	D	98
G 278	20	48	29	4,032	D + 1	D	?
G 284	10	26	25	9,990	D + 1	D	48
G 288	13	25	16	40	D	D − 9	56
G 294	7	17	14	7,344	D + 4	D + 1	112
G 295	7	17	14	25,920	D + 4	D + 1	52
G 302	13	22	15	14,832	D + 2	D	65
G 307	11	35	8	500	D + 2	D − 24	85
G 312	10	16	10	13,800	D + 4	D + 1	86
G 319	8	23	14	6,720	D + 2	D	97
G 323	8	11	6	14,688	D + 1	D + 2	79
G 325	7	22	13	4,480	D	D	297
G 326	7	29	17	9,900	D + 1	D + 1	76
G 330	9	21	14	4,290	D + 1	D	83
G 332	12	27	14	7,920	D + 1	D	92
G 369	14	24	17	1,120	D + 1	D	100
G 374	7	44	13	3,144	D + 1	D − 10	176
G 404	11	30	18	28,800	D + 3	D + 1	87
G 406	15	25	13	14,688	D + 3	D	88
G 411	18	47	23	3,960	D + 1	D + 1	88
G 412	6	25	16	10,440	D + 2	D	380
G 420	27	17	7	9,648	D + 2	D + 1	86
G 422	11	17	13	4,968	D + 3	D	?
G 437	10	29	15	10,800	D + 2	D + 1	?
G 440	12	26	15	2,160	D + 1	D + 1	123
G 453	9	23	11	9,072	D + 1	D	?
G 459	9	22	14	20,160	D + 2	D + 1	87

TABLE 1—*Continued*

Patient no.	No. of infective mosq. bites	Day of parasite patency	Previous no. of paroxysms	Parasites per cmm. at R_x	Last parasite day*	Last day fever 100°*	Relapse: days after treatment‡
G 461.....	3	27	15	7,020	D + 2	D − 1	?
G 464.....	11	30	16	5,568	D + 3	D − 1	95
G 465.....	6	23	17	26,640	D + 2	D	?
G 476.....	3	24	15	20,700	D + 2	D + 1	98
G 483.....	7†	11	8	18,900	D + 3	D − 1	120
G 486.....	8†	26	12	1,890	D + 1	D	?
G 489.....	10†	41	16	3,030	D	D − 1	?
Median....	10	23	14	10,620	D + 2	D	94

* D is day of treatment.

† No. of infected mosquito salivary glands preserved by freezing and inoculated intravenously.

‡ Days after first day of treatment.

? Indicates no data available.

gram base) were followed in six hours by one tablet (0.3 gram base); on the second and third days one tablet (0.3 gram base) each day concluded the course. In a smaller number of cases, chloroquine was administered intramuscularly as the hydrochloride in sterile aqueous solution; a single dose of 0.4 gram base consituted the treatment.

The group of patients of primary interest consisted of white patients who gave no history of previous *vivax* malaria infection. Two minor groups are also included in the study: ten white patients who had previously experienced the Chesson strain of *vivax* and possessed considerable homologous immunity, and two Negro patients in whom infections were produced but whose infections differed considerably from those considered normal in white patients.

RESULTS

Nearly 100 cases were included in the study; however, incomplete data on some of these eliminated them from consideration. The results will be considered in several sections and will include the treatment of 83 primary attacks and 101 relapses (first through fifth).

Treatment of primary attacks. Fifty-eight uncomplicated primary attacks were treated with 1.5 grams of chloroquine orally (Table 1) and 13 with 0.4 gram intramuscularly (Table 2); only 45 of the former and 8 of the latter were observed for relapses. In Table 1 is summarized the effect of 1.5 grams oral chloroquine on 58 primary attacks; the drug was administered after 6 to 71 days of parasite patency and 3 to 32 febrile episodes. Parasitemias at time of treatment ranged from 40 to 82,800 per cmm.; the parasitemias were eliminated in 24 to 144 hours. If treatment was during the clinical stage of the infection fever was eliminated within 24 or 48 hours in 90 per cent of the cases but persisted for as long as 72 or 96 hours following initiation of treatment in about 10 per cent. All cases followed for a sufficiently long period were found to relapse after treatment.

TABLE 2

Treatment of P. vivax *(Chesson) primary attacks with 0.4 grams chloroquine base intramuscularly*

Patient no.	No. of infective mosq. bites	Day of parasite patency	Previous no. of paroxysms	Parasites per cmm. at R_x	Last parasite day*	Last day fever 100°*	Relapse: days after treatment
G 255	5	11	7	41,760	D + 1	D	?
G 298	2	31	18	4,848	D + 1	D + 2	?
G 337	22	9	6	7,416	D + 2	D	73
G 343	10	23	16	430	D + 1	D	37
G 347	7	9	4	18,180	D + 2	D	?
G 348	7	14	9	7,920	D + 2	D	138
G 351	9	9	6	4,000	D + 2	D + 2	85
G 353	3	24	19	7,776	D + 1	D	85
G 370	18	39	13	6,900	D + 1	D − 1	87
G 379	23	15	11	16,416	D + 1	D	?
G 392	10	22	8	13,050	D + 3	D	89
G 401	14	25	8	22,680	D + 4	D + 1	?
G 450	20	25	17	4,500	D + 1	D	145
Median	10	22	9	7,776	D + 1	D	93

* D is day of treatment.

? Indicates no data available.

TABLE 3

Relation of the number of febrile episodes (paroxysms) prior to treatment of P. vivax *(Chesson) with 1.5 grams chloroquine base to the length of latency following treatment. (46 cases)*

No. of cases	Number of paroxysms		Days from treatment to relapse	
	Range	Median	Range	Median
17	3–10	8	43–149	86
22	13–20	15	52–380	95
7	22–32	23	48–322	95

The period intervening between the treatment of the primary attack and renewed parasite activity varied from 43 to 380 days, with a median of 94 days.

In Table 2, 13 primary attacks were treated with 0.4 gram chloroquine intramuscularly after 9 to 39 days of patent parasites and 4 to 19 febrile episodes. Parasitemias at time of treatment ranged from 430 to 41,760 per cmm.; the parasitemias were eliminated in 48 to 120 hours and fever in 24 to 72 hours after initiation of drug. In all cases where observations were continued for a sufficiently long period, relapses were seen. These occurred 37 to 145 days (median 86) after treatment.

In an effort to relate the length of latency following treatment of the primary attack (1.5 grams chloroquine) with the severity or duration of the primary attack, the relapsing cases were grouped according to the duration of parasitemias and number of febrile episodes prior to treatment. Table 3 suggests that the

TABLE 4

Relation of the number of days of patent parasitemia prior to treatment of P. vivax *(Chesson) with 1.5 grams chloroquine base to the length of latency following treatment (46 cases)*

No. of cases	No. of days of patent parasitemia		Days from treatment to relapse	
	Range	Median	Range	Median
6	6–10	8.5	59–149	102.5
14	11–19	15.5	43–120	86
21	21–30	25	48–380	95
5	31–71	44	85–322	103

number of paroxysms has little effect on the length of latency following treatment of the primary attack. The same may be said of the number of days of patent parasitemia (Table 4) during the primary attack. Individual comparisons can be made in Tables 1 and 2, noting that comparatively short periods of parasitemia and low numbers of paroxysms are frequently followed by long latent periods, while the reverse may be true in other cases.

Treatment of relapses. Relapses were treated with chloroquine, either 1.5 grams orally or 0.4 gram intramuscularly, and were generally terminated within a few days of their detection. In a few cases the relapsing parasitemias and paroxysms were allowed to continue for longer periods before treatment. Table 5 summarizes treatment and relapses in 31 cases which were followed through more than one relapse. In two of the cases the primary attacks were terminated with chlorguanide rather than chloroquine, but these are included because subsequent attacks were treated with chloroquine.

Twenty-five of 30 cases in which the second attack (first relapse) was treated with chloroquine relapsed after 30 to 284 days (median: 85 days). The five cases which failed to relapse were probably followed for sufficient periods, although two of the cases were followed for periods less than the longest period of latency seen in one of the relapsing cases. One second attack (G 292) was not treated and terminated spontaneously after a few days of parasites; in this case the third attack occurred within a relatively short time (26 days). Relapses seemed to occur no sooner after treatment of the second attack with 0.4 gram intramuscularly than they did following 1.5 grams orally. Periods of 30 to 231 days (median: 86) followed the 0.4 gram dosage and 48 to 284 days (median: 85 days) followed the 1.5 grams regimen.

Seventeen of 19 cases in which the third attack was treated with chloroquine relapsed after 26 to 313 days (median: 80 days). The two which failed to relapse were followed for 186 and 378 days negative, and it is possible that the former had not concluded activity at that time. Again relapses seemed to occur no sooner following 0.4 gram of chloroquine than they did following the 1.5 grams regimen.

Seven of ten cases where the fourth attack was treated with chloroquine relapsed after 69 to 181 days (median: 80 days). The three cases which failed to relapse were followed 149, 155, and 264 days after treatment, probably sufficient lengths of time, although in one of the cases relapsing parasitemias did not appear until 181 days after treatment.

TABLE 5

P. vivax (*Chesson strain*): *Relapses after treatment of first, second, third and fourth attacks with chloroquine*

Patient no.	Treatment of attack #1	Relapse: days after R_x	Treatment of attack #2	Relapse: days after R_x	Treatment of attack #3	Relapse: days after R_x	Treatment of attack #4	Relapse: days after R_x	Last day of parasites*
G 55...	Chlor. 1.5 g.	49	Chlor. 1.5 g.	87	Chlor. 1.5 g.	26	Chlor. 1.5 g.	None to 155	216
G 112...	Chlor. 1.5 g.	43	Chlor. 1.5 g.	48	Chlor. 1.5 g.	55	Chlor. 1.5 g.	69	301
G 126...	Chlor. 1.5 g.	78	Chlor. 1.5 g.	91	Chlor. 1.5 g.	Dropped			186(?)
G 175...	Chlor. 1.5 g.	123	Chlor. 1.5 g.	79	Chlor. 1.5 g.	Dropped			210(?)
G 213...	Chlor. 1.5 g.	105	Chlor. 1.5 g.	110	Nil	Dropped			418(?)
G 244...	Chlor. 1.5 g.	112	Chlor. 1.5 g.	None to 363					174
G 267...	Chlor. 1.5 g.	59	Chlor. 1.5 g.	106	Chlor. 1.5 g.	236	Chlor. 1.5 g.	None to 149	462
G 276...	Chlor. 1.5 g.	98	Chlor. 1.5 g.	66	Chlor. 1.5 g.	76	Chlor. 1.5 g.	85	815
G 284...	Chlor. 1.5 g.	48	Chlor. 1.5 g.	51	Chlor. 1.5 g.	62	Chlor. 1.5 g.	70	291
G 288...	Chlor. 1.5 g.	56	Chlor. 1.5 g.	85	Nil				1422
G 307...	Chlor. 1.5 g.	85	Chlor. 1.5 g.	142	Chlor. 1.5 g.	72	Chlor. 1.5 g.	76	427(P)
G 326...	Chlor. 1.5 g.	76	Chlor. 0.4 g.	53	Chlor. 0.4 g.	96	Chlor. 0.4 g.	181	513
G 325...	Chlor. 1.5 g.	297	Chlor. 1.5 g.	None to 205					319
G 323...	Chlor. 1.5 g.	79	Chlor. 0.4 g.	79	Chlor. 1.5 g.	None to 378			175
G 332...	Chlor. 1.5 g.	92	Chlor. 1.5 g.	59	Chlor. 1.5 g.	109	Nil		364
G 369...	Chlor. 1.5 g.	100	Chlor. 1.5 g.	105	Chlor. 1.5 g.	137	Nil		405
G 374...	Chlor. 1.5 g.	176	Chlor. 1.5 g.	284	Chlor. 1.5 g.	149	Nil		849
G 406...	Chlor. 1.5 g.	88	Chlor. 1.5 g.	58	Chlor. 1.5 g. + Prim.	Dropped			189(P)
G 412...	Chlor. 1.5 g.	380	Chlor. 1.5 g.	None to 509					411
G 420...	Chlor. 1.5 g.	86	Chlor. 1.5 g.	74	Chlor. 1.5 g.	65	Nil		492
G 459...	Chlor. 1.5 g.	87	Chlor. 1.5 g.	73	Chlor. 1.5 g.	None to 186			232
G 464...	Chlor. 1.5 g.	95	Chlor. 1.5 g.	140	Chlor. 0.4 g.	53	Chlor. 1.5 g.	Dropped	336(?)
G 337...	Chlor. 0.4 g.	73	Chlor. 0.4 g.	30	Chlor. 1.5 g.	47	Nil	Dropped	289(?)
G 343...	Chlor. 0.4 g.	37	Chlor. 1.5 g.	63	Chlor. 1.5 g.	80	Chlor. 1.5 g.	83	299(P)
G 348...	Chlor. 0.4 g.	138	Chlor. 1.5 g.	None to 298					172
G 351...	Chlor. 0.4 g.	85	Chlor. 0.4 g.	96	Prim. + Quinine	Dropped			200(P)
G 370...	Chlor. 0.4 g.	87	Chlor. 1.5 g.	153	Chlor. 1.5 g.	Dropped			285(?)
G 392...	Chlor. 0.4 g.	89	Chlor. 0.4 g.	231	Chlor. 0.4 g.	313	Chlor. 0.4 g.	Dropped	655(?)
G 450...	Chlor. 0.4 g.	145	Chlor. 0.4 g.	None to 262					191
G 339...	Palud. 0.1 g.	152	Chlor. 0.4 g.	93	Chlor. 0.4 g.	129	Chlor. 0.4 g.	None to 264	394
G 292...	Palud. 0.4 g.	73	Nil	—	Chlor. 1.5 g.	158	Chlor. 1.5 g.	67	368(P)

Chlor. 1.5 = 1.5 grams base given orally over a 3-day period.
Chlor. 0.4 = 0.4 gram base given intramuscularly in single dose.
Palud. 0.4 = 0.4 gram chlorguanide given orally 100 mg. per day (87% base).
Palud. 0.1 = 0.1 gram chlorguanide given orally in single dose (87% base).
When treatment is listed as "Nil", the parasitemias and symptoms undergo spontaneous remission after varying periods of presence.
* No. of days after first patency.
(P) indicates treatment with primaquine may have prevented further parasite activity.
(?) may have experienced further parasite activity.

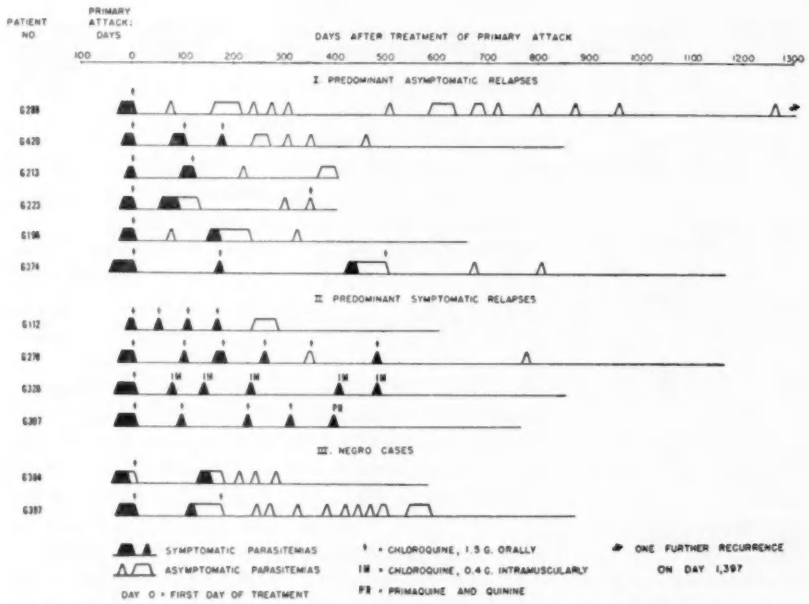

FIG. 1. Course of infection in twelve chloroquine-treated cases of *P. vivax* (Chesson).

Of the seven patients experiencing fifth attacks, three were terminated with curative drug (primaquine), two terminated spontaneously without drug, and two were treated with chloroquine for a fifth time, one (G 326) with 0.4 gram and the other (G 276) with 1.5 grams. Both of these cases experienced a sixth attack, after 66 and 117 days, respectively, and were again treated with the same chloroquine dosages. No further relapses were seen in G 326, although observations were continued for 367 days after last treatment. Case G 276 showed patent parasites on a single day 297 days after last treatment, but no further parasitemias were seen during a final observation period of 370 days.

In a number of cases the relapsing parasitemias were asymptomatic or accompanied by only transient symptoms and were allowed to terminate spontaneously, without administration of drug. Frequently in these cases asymptomatic parasitemias recurred at variable intervals for considerable periods of time. Six of the cases of long duration (I) are illustrated in Figure 1, with a number of the cases experiencing symptomatic relapses and chloroquine treatment (II) included for comparison. Also illustrated in Figure 1 (III) are two infections in Negro patients.

The parasitema of longest duration was seen in case G 288; patent parasitemia occurred as late as 1397 days after treatment of the primary attack. It will be noted that in this case no symptoms accompanied the frequent recurrence of parasites during the whole course of infection. In the other cases illustrated, symptoms did accompany the earlier parasite recurrences. In most cases the

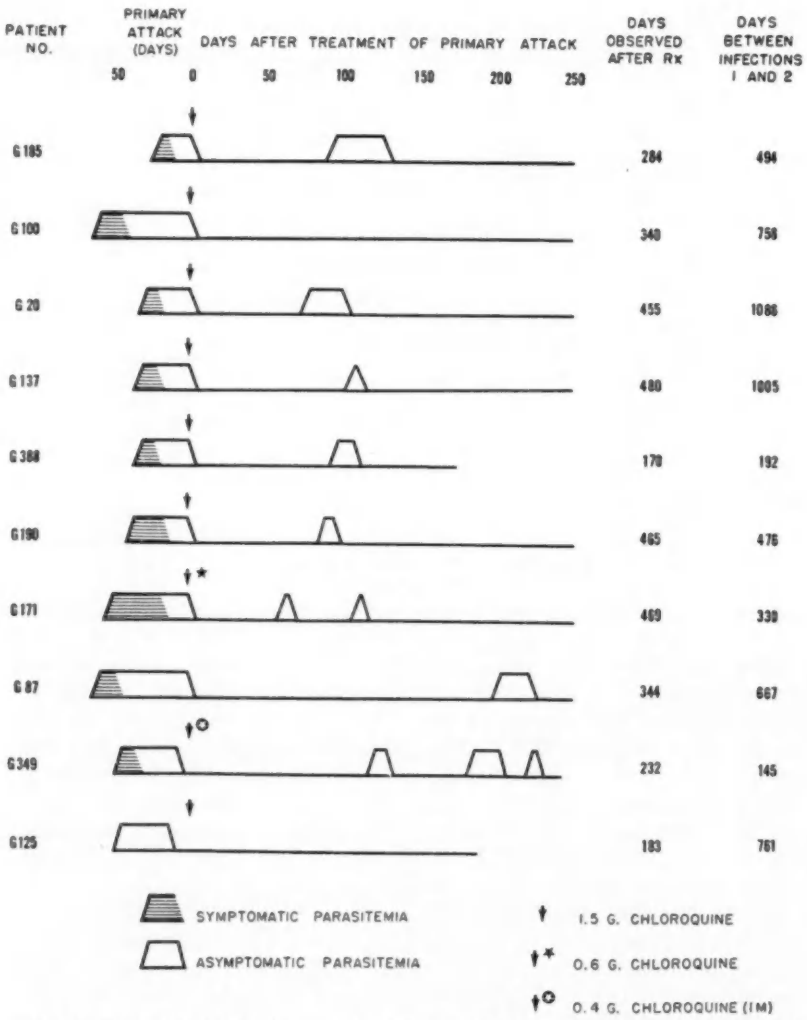

FIG. 2. Course of infection in ten cases of *P. vivax* (Chesson) who previously had experienced the homologous strain.

asymptomatic parasitemias were preceded by second or third attacks which were allowed to run for considerable lengths of time before they were terminated, either by drug or spontaneously. On the other hand in those cases in which the second and third attacks were terminated promptly by drug, further relapses were usually symptomatic as long as they occurred.

The two Negro cases show a pattern of parasite recurrences somewhat different from those previously described. In one of these cases (G 364) the primary parasitemias became asymptomatic before termination by drug. In spite of this the second attack began as a symptomatic relapse, though it soon became asymptomatic. The same was true of the second attack of G 397. Following this symptomatic period both cases became asymptomatic and showed intermittent parasitemias for a considerable length of time.

It can be determined from Tables 1 and 5 that the number of infective mosquito bites used in the inoculation of the patients did not have a consistent effect on the relapse pattern or on the total duration of the infection. The median number of bites used for inoculations was ten and, while there were no single bite inoculations, there was a sufficient range to indicate the absence of any obvious correlation in these respects.

Treatment of partially immune patients. In ten cases (Fig. 2) the infection was induced by mosquito bites in patients who had previously experienced the homologous parasite, eight by blood inoculation and two by mosquitoes (G 388 and G 125). The period between termination of the initial infection and induction of the second varied from 145 to 1,086 days. In nine cases the second inoculation produced a transitory clinical attack; no symptoms were seen in the tenth case (G 125). Chloroquine was given in nine cases and recurrent asymptomatic parasitemias were encountered in eight of the nine. These parasitemias usually occurred only a single time and were of low density. The severity of the primary attack or the relapse pattern seemed to bear little relationship to the length of the interval between the immunizing and second infection. It is apparent that considerable tolerance to the parasite resulted from the initial infection and persisted in several cases for nearly three years after termination of the initial infection.

DISCUSSION

The rapid effect of chloroquine on the clinical attack compares favorably with the observations of Most *et al.*, (1946), Pullman *et al.*, (1948), Culwell *et al.*, (1948), Young and Eyles (1948), and others. In our cases there seemed to be no important difference between the effect of the oral and intramuscular dosages. Parasitemias and fever were perhaps eliminated somewhat earlier by the parenteral administration of the drug.

Despite the numerous investigations which have been done on chloroquine as an antimalarial, there is little in the literature concerning the relapse pattern of the Chesson type of *P. vivax* after treatment. Pullman *et al.*, (1948) treated eight volunteers (two primary attacks and six relapses) with two grams of chloroquine over a seven-day period. Relapsing parasitemias appeared 45 to 236 days after cessation of treatment, with a median interval of about 60 days. Except for the one case where the interval was unusually long (236 days), 68 days was the longest intervening period. Coatney, Cooper, and Young (1950) report three cases treated with 1.5 grams chloroquine in four days, and four with 6.0 grams in 14 days. In the former group relapses occurred 42, 49, and 50 days

after treatment. Subsequent attacks occurred after 49, 58, 98, and 121 days when treatment was with the same regimen. When the larger dosage of chloroquine was used relapses were delayed somewhat, first relapses occurring after 75, 81, 87, and 87 days, second relapses after 61 to 86 days and third relapses (in three cases) after 129 to 136 days. Although it is not specifically stated, it is presumed that the subjects were observed for a sufficient length of time for detection of all relapsing parasitemias.

The latter investigators also have reported a number of cases where primary and subsequent attacks were treated with single doses of 0.3 gram chloroquine orally. Parasitemias recurred after relatively short latency in these cases, but the dosage is too near the minimum necessary for the eradication of erythrocytic parasites to be certain of the status of the recurring parasitemias.

Culwell *et al.*, (1948) reported the treatment of Chesson *vivax* attacks in volunteers with intramuscular chloroquine. Only five patients were treated with dosages comparable to those in the present study and these were second attacks. Clearance of parasitemia and fever was prompt and parasitemias recurred after intervals of 27 to 42 days (median: 34 days).

It appears that where the intervals between attacks of chloroquine-treated Chesson *vivax* are concerned there is considerable variance between our observations and those of others. The explanation of these differences is fairly obvious. The cases reported by others were in volunteers who generally received drug after a minimal amount of clinical malaria and parasitemia. In the present study the malaria had been induced as part of treatment for neurosyphilis and the patient was allowed to experience as much malaria, at least during the primary attack, as his physical condition or other considerations would allow. Occasionally this even applied to subsequent attacks. Apparently immunological phenomena have a profound effect on the intervals between attacks and on the frequency of relapses. Introduction of this factor or set of factors necessarily complicates the situation considerably and the individual variations between patients and their response to malaria infection become important. This variation in individual response to the infection undoubtedly is responsible for the wide variation in intervals between attacks and in the overall duration of the infection. Our results indicate that within the group of patients who were allowed to experience clinical malaria of even a fairly short duration (as low as three paroxysms) the actual numbers of febrile episodes or days of parasitemia did not have a vast effect on the intervals. In general, it is apparent that the clinical course delayed the reappearance of parasites after treatment considerably over the intervals which were seen after early termination of the infection. Coatney *et al.* (1950) note the progressive increase in treatment-to-relapse intervals in the same patient and suggest that this may be due to a gradual development of immunity to erythrocytic parasites. In our cases this development of immunity may be accomplished during the primary attack, thereby producing a relatively long interval earlier in the course of infection.

Further indication that the treatment-to-relapse interval is a function of the immunity rather than dosage or excretion rate of drug (Coatney *et al.*, 1950)

can be seen in the similarity in these intervals in the attacks treated with 1.5 grams orally and those with 0.4 gram intramuscularly. However, in cases where immunity was not a factor (Coatney *et al.*) the use of 6.0 grams of chloroquine produced a much longer treatment-to-relapse interval than did 1.5 grams.

Of interest in this same regard are three primary attacks which were treated with low dosages of chlorguanide. Two of these cases are listed in Table 5, because their subsequent treatment was with chloroquine. Treatment of primaries with 0.1 gram and 0.4 gram (87 per cent base) resulted in a latent period of 152 days and 73 days, respectively. A third case treated with 0.4 gram relapsed 90 days after treatment. These latent periods compare well with those seen after 1.5 grams of chloroquine and are considerably longer than those seen by Coatney *et al.* (1950), even though they used a total dose of 14 grams of chlorguanide. In four such treatments of the primary attacks, they found renewed parasite activity after 21 to 25 days. Here again it is obvious that dosage of drug had little to do with the subsequent relapse.

The relapse pattern or the overall duration of the infection in our cases could not be related to the infective sporozoite dosage. Coatney *et al.* (1950), Boyd (1940), and Craige *et al.* (1947) suggest that such infective inoculum may be of significance in the subsequent malarial activity. Our cases covered a wide range of infective bites per patient (3 to 100), and we saw no significant evidence of effect on the subsequent infection. Neither could we note any correlation between the length of the prepatent period, as an evidence of infective dosage, and the subsequent relapse pattern. This differs from the results of Craige *et al.* (1947), but in our cases only a single prepatency of more than 14 days was seen (21 days), while Craige *et al.*, divided the cases into groups with prepatent period of 14 days and under and 15 days and over, finding the two groups to differ significantly in relapse rates and in length of latency before relapse.

Of considerable interest are the immune cases reinoculated with the homologous parasite. These cases almost invariably experienced some symptoms early in the primary attack and also almost invariably experienced one parasite recurrence, always asymptomatic. One might speculate as to the pattern of malarial activity which would result from addition of a typical case in an immune to the pattern of the initial infection (Figure 1). With frequent addition of this type of infection to the pattern one might picture the long-term pattern in a given resident of an area of high endemicity. Immunity would prevent the patient who survived his primary attack from being too ill during subsequent attacks and infections but would not prevent continual reinfection, with only mild or short-lived symptoms.

There is, of course, the question as to the epidemiological significance of these recurrent parasitemias: are they important in the propagation of the infection? Eyles, Young, and Burgess (1948) were able to infect mosquitoes on asymptomatic, as well as symptomatic, parasitemias of Pacific (Chesson type) *P. vivax*. Most of the infections obtained from asymptomatic parasitemias resulted from pre-clinical and terminal (post-clinical) periods, but in two of three trials infections were obtained on interval asymptomatic parasitemias. Unpublished data

which we have collected on the Chesson strain indicate that whenever relapsing parasitemias are accompanied by symptoms they will generally infect mosquitoes. Further, the infectivity to mosquitoes frequently extends into the pre- and post-clinical periods of parasitemia, which confirms the results of Eyles *et al.* (1948). However, in this strain we have not been able to infect mosquitoes with recurrent parasitemias which were not accompanied by malarial symptoms, these parasitemias corresponding to the "interval asymptomatic parasitemias" of Eyles *et al.* Thus it can be seen that, although some uncertainty exists in regard to parasitemias unaccompanied by symptoms, many of the recurrent parasitemias are of importance in perpetuation of the infection.

Coatney *et al.* (1950), in their summary of 204 sporozoite induced infections with Chesson *vivax* concluded that "this study has re-emphasized the variability of the behavior of *vivax* malaria under conditions where many factors were controlled. Under uncontrolled natural conditions the pattern of relapsing *vivax* malaria certainly can be more complex". It is believed that the present study confirms this conclusion. The cases included can undoubtedly qualify as being more like the "uncontrolled natural conditions" specified, and certainly the pattern of infection was infinitely more complex.

SUMMARY AND CONCLUSIONS

Observations are reported on a group of nearly 100 patients with sporozoite-inoculated *Plasmodium vivax* (Chesson strain) treated with chloroquine. Included was the treatment of 83 primary attacks and 101 relapses. Two treatment regimens were used: 1.5 grams base orally or 0.4 gram base intramuscularly. The primary attacks responded rapidly to treatment with either regimen. All cases followed sufficiently long were found to relapse after treatment. Of these, 45 were treated with 1.5 grams and 8 with 0.4 gram; there was no appreciable difference between the two where the treatment-to-relapse interval was concerned. The severity or duration of the primary attack prior to treatment could not be related to the treatment-to-relapse interval, nor could the number of infective bites used to induce the infection.

Relapses were treated as they occurred with one of the two regimens and were followed to further relapse or to conclusion in a number of cases. Treatment-to-relapse intervals were about the same after each attack; there was no difference in intervals after 1.5 or 0.4 grams dosages of drug. Twenty-five of 30 first relapses showed renewed activity after treatment, as did 17 of 19 second relapses, 7 of 10 third relapses, and two of four fourth relapses. One of the fifth relapses showed renewed activity.

Immunes inoculated with sporozoites usually experienced a short clinical course and, after treatment, a single asymptomatic parasite relapse.

Relapses occurred later and less frequently after treatment of extended clinical attacks than was the case in those attacks terminated prior to extensive clinical malaria, as reported by others in experimental work in volunteers.

It is concluded that in the Chesson strain of *P. vivax* extreme variability in patterns of infection and relapse is the rule in cases of the type considered.

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FURTHER STUDIES ON CROSS-RESISTANCE BETWEEN PYRIMETHAMINE AND RELATED COMPOUNDS

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It was previously reported (Greenberg and Bond, 1954) that a strain of *Plasmodium gallinaceum* made 64-fold resistant to pyrimethamine was 2-fold resistant to certain analogues of pyrimethamine and up to 512-fold resistant to others. The strain was more resistant to 5-phenyl analogues of pyrimethamine than it was to 5-benzyl or 5-phenoxy analogues, or to a 5-phenyltriazine. Chicks can tolerate a larger amount of pyrimethamine than the amount to which the parasite had been made resistant. By exposing the 64-fold resistant strain of parasites to increasing amounts of the drug, a strain of *P. gallinaceum* has now been derived which is more than 800-fold resistant to pyrimethamine. Most of the compounds tested in the previous report have been retested against the new strain in order to determine whether any significant changes in resistance to any of them had occurred.

MATERIALS AND METHODS

Week-old New Hampshire red chicks were used as hosts. The strain of *Plasmodium gallinaceum* used in the present experiments was derived from a strain made 64-fold resistant to pyrimethamine as described by Greenberg and Bond (1954). Resistance to the drug was increased by exposing the parasite in the chick to gradually increasing amounts of the drug over a period of about six months. The drug was administered twice daily for four days beginning four to five hours before the intravenous inoculation of 16 million parasitized erythrocytes. When the mean parasitemia of five chicks examined on day 4 exceeded 25 per cent of that in untreated controls, the dosage of the drug in the next passage was doubled. A three-day drug-free period often intervened between passages. The strain eventually became more than 800-fold resistant to pyrimethamine as compared with a normal sensitive strain and 12-fold more resistant than its immediate parent strain.

The activity of the test compounds was determined by methods described in detail elsewhere (Coatney *et al.*, 1953). Week-old chicks were inoculated with 16 million parasitized erythrocytes and were treated twice daily for four days beginning 4 to 5 hours before inoculation. Parasitemia was determined on the morning of day 4 (day of inoculation is day 0). Five chicks were used in each treated group and for a series of such groups ten untreated chicks served as controls. The minimum effective dose of a compound is the smallest dose which produces a mean 75 per cent reduction in parasitemia in treated, as compared with untreated, chicks.

We are grateful to Burroughs Wellcome & Co. for supplying the 2,4-diamino-

TABLE 1

The effect of diaminopyrimidines, chlorguanide, and a diaminotriazine on two pyrimethamine-resistant strains of Plasmodium gallinaceum

Compound	Minimum effective dose mg./gm. b.i.d. × 4 days		
	Parent strain*	Resistant strain 1*	Resistant strain 2
DR 16056	0.00004	0.00256	>0.032 t
DR 16311	0.00008	0.00128	>0.0225 t
DR 16093	0.0000625	0.032	>0.128 t
DR 16071	0.001	0.008	>0.128 t
DR 16092	0.001	0.002	>0.064 t
DR 15787	0.0078	0.0156	>0.248 t
SN 12837	0.002	0.032	>0.064 t
DR 16182	0.00002	0.00016	0.00512

* Data from Greenberg and Bond, 1954.
DR 16056—Pyrimethamine: 2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine.
DR 16311—2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine.
DR 16093—2,4-Diamino-5-(*p*-chlorophenyl)-6-amylpyrimidine.
DR 16071—2,4-Diamino-5-(*p*-chlorobenzyl)-6-methylpyrimidine.
DR 16092—2,4-Diamino-5-(*p*-nitrobenzyl)-6-methylpyrimidine.
DR 15787—2,4-Diamino-5-(*p*-chlorophenoxy)-6-methylpyrimidine.
SN 12837—Chlorguanide: N_1-(*p*-chlorophenyl)-N_5-isopropylbiguanide
DR 16182—4,6 - Diamino - 1 - (3′,4′ - dichlorophenyl) - 1,2 - dihydro - 2,2 - dimethyl - 1,3,5-triazine
t = maximum tolerated dose.

pyrimidines and to Imperial Chemical Industries for chlorguanide and the triazine.

RESULTS

The minimum effective dose of the eight compounds tested in the present series of experiments is shown in Table 1, together with results previously obtained with the original sensitive parent strain and the 64-fold resistant strain. When the strain was made more than 800-fold resistant to pyrimethamine, resistance to all the other compounds increased significantly, but the exact degree of resistance could not be determined (except with DR 16182) because the compounds had no effect on the parasites at the highest dose tolerated by the chicks. DR 16182 was effective at the maximum tolerated dose. Especially noteworthy is the fact that the resistance to DR 16092 and DR 15787 had increased from 2-fold to more than 64-fold and 32-fold, respectively.

DISCUSSION

Pyrimethamine and related compounds offer a unique opportunity in studies such as these. Resistance to one of these compounds (pyrimethamine) can be readily induced (Jaswant Singh *et al.*, 1952; Schmidt and Genther, 1953; Hernandez *et al.*, 1953; Thurston, 1953). Because of the high therapeutic indices of some of the compounds (500 to 1000), the number of distinct stepwise increments in resistance is large. A large number of these compounds have already been

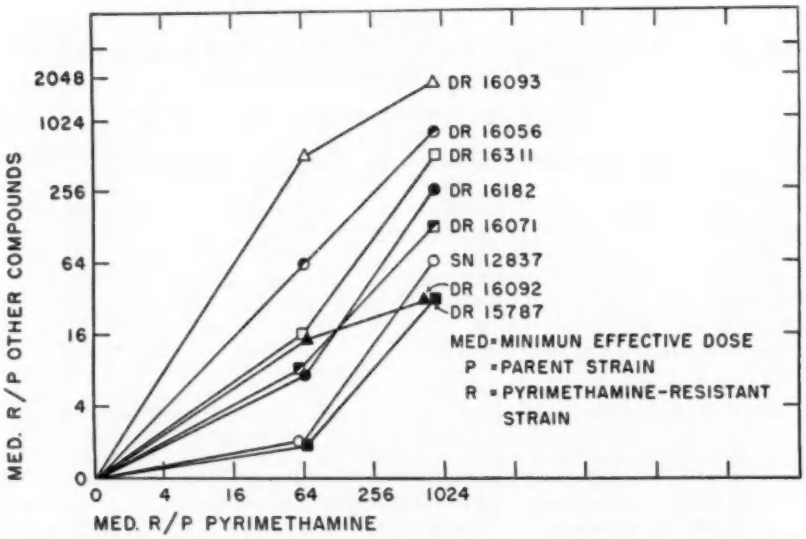

FIG. 1. The degree of resistance to certain diaminopyrimidines, chlorguanide, and a diaminopyrimidine of a strain of *Plasmodium gallinaceum* at three stages of resistance to pyrimethamine.

synthesized and tested for antimalarial activity, and many are extremely active (Falco *et al.*, 1951).

The present study and that reported earlier (Greenberg and Bond, 1954) indicate that cross-resistance among chemically related, and presumably biologically related, compounds is a complicated phenomenon. As the resistance of the parasite to pyrimethamine increased to 64-fold, its resistance to the 5-*p*-chlorophenoxy (DR 15787) and 5-*p*-nitrobenzyl (DR 16092) analogues increased about 2-fold. A subsequent 12.5-fold increase in resistance to pyrimethamine was associated with a marked increase of resistance to these same compounds (16-fold and 32-fold, respectively). The data have been plotted with the three points now available for each of the compounds tested (Figure 1). The points to the right on this graph are uncertain because of the limitations of the host tolerance for the drugs. These data may actually be an underestimate of the degree of resistance of the parasites to the drug. Even so, the slope of the curves as the parasite progressed from no resistance to 64-fold resistance was different among most of the compounds. Furthermore, the slope of the curve from zero to 64-fold pyrimethamine-resistance is different in most compounds from the curve between 64- and 800-fold resistance to pyrimethamine.

The simplest conclusion to be drawn from the data is that as parasites become more resistant to one member of an analogous series, they are more likely to become resistant to related compounds. These observations may explain why in some reports there was cross-resistance between chlorguanide and pyri-

methamine and in others there was not. But much is left unexplained, especially the lack of proportional increase in resistance throughout the gradient curve. Aside from the fact that the final point is not firm for most compounds because of the limitation of host tolerance, it is not certain whether the increase in resistance to these compounds encountered as the parasite became between 64- and 800-fold resistant to pyrimethamine occurred gradually or suddenly. It is conceivable that no change in resistance to DR 15787 occurred until the parasite became 800-fold resistant to pyrimethamine. This point might be worth further inquiry. It might also be worth doing the reciprocal experiments in which the parasite was gradually made resistant to DR 15787 and tested for its resistance to pyrimethamine.

In our last report it was suggested that resistance to pyrimethamine was probably not mediated by shunting of certain sensitive biological processes to other pathways. It was suggested that increase in resistance was the result of small, sequential changes in a cell receptor which rejected the inhibitor. Early in such a series of changes, compounds which are closely related chemically might be rejected preferentially. Subsequent changes, however, might be sufficiently large so that less closely related chemicals might also be rejected. If one postulates a pyrimethamine-destroying enzyme as an explanation for developing resistance, then by small changes this enzyme could accept less and less closely related chemicals for destruction as resistance increases. Alternatively, changes in another sensitive portion of the molecule might affect compounds previously not affected. Substituted metabolic pathways or other explanations for increased resistance cannot be completely rejected since these might constitute one step in a large series of changes. Such a step might account for the sudden increase in resistance to other compounds that were previously little affected by the increase in resistance to pyrimethamine.

SUMMARY

A strain of *Plasmodium gallinaceum* was made 800-fold resistant to pyrimethamine. The strain was found to be resistant to several other 2,4-diaminopyrimidines, chlorguanide, and a diaminotriazine at the highest dose tolerated by the chicks. These data are compared with those obtained earlier when the strain was only 64-fold resistant to pyrimethamine and these differences are discussed.

ACKNOWLEDGEMENTS

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DIFFERENCES IN THE COURSE OF *PLASMODIUM BERGHEI* INFECTIONS IN SOME HYBRID AND BACKCROSS MICE

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In earlier studies it was found that (1) inbred strains of mice differed in their resistance to malaria as determined by their survival after inoculation with *Plasmodium berghei* (Greenberg *et al.*, 1953, 1954); (2) F_1 hybrids of these inbred strains usually survived longer than either parent (Greenberg *et al.*, 1953, 1954; Nadel *et al.*, 1955; Greenberg and Coatney, 1955; Highman *et al.*, 1955); and (3) the backcross of one hybrid (C 57 Black × DBA) to the shorter-lived parent (DBA) was intermediate in survival between the two parents while the backcross to the longer-lived parent (C 57 Black) lived longer than either parent (Nadel *et al.*, 1955). Studies on the pathological changes induced by the parasite (Highman *et al.*, 1955) in short-lived white Swiss mice and long-lived C 57 Leaden × A (LAF) hybrids, revealed the following: (1) The onset of pathological lesions was earlier and the lesions were more severe in the Swiss mice, (2) The cause of death in the short-lived mice was most likely toxemia; anoxia was the most probable cause of death in the long-lived mice. (3) There were differences in the degree to which mature and immature (polychromatophilic) erythrocytes were invaded in the two groups of mice. In regard to the last point, about 84 per cent of the mature erythrocytes of the white Swiss mice were infected on day 8 after inoculation; in corresponding LAF mice only 24 per cent of the mature erythrocytes were infected. The infection of mature erythrocytes declined in LAF mice after day 8 until by the end of the third week only 12 per cent of these cells were infected. The infection of immature erythrocytes was high on the eighth day and continued high until the death of the mice. The proportion of immature to mature erythrocytes increased after the first week until the immature cells constituted about 56 per cent of the population. The infection during the third week in LAF mice was predominantly in immature erythrocytes.

It appeared that there might be some relationship between the degree of infection of mature erythrocytes at the end of the first week and the probability that the mice might die at this time of toxemia. This possibility has been explored in four types of mice which had, in previous experience, distinct differences in mean survival after inoculation with *P. berghei*: (1) white Swiss (about 8 days); (C 57 Black × DBA) × DBA backcross (about 13 days); LAF (about 20 days); and STR × C 57 Black (about 25 days).

MATERIALS AND METHODS

The Kasapa strain of *Plasmodium berghei* was used throughout. This strain was maintained by weekly subinoculation of parasitized blood in general purpose white Swiss mice. White Swiss mice served as donors for all the experiments,

TABLE 1

The age and sex of mice used in the present study

Type of mouse	Age of groups (months)	Sex
White swiss	1½, 2, 2, 2½, 4, 6	5♂ 20♀
C57 leaden × A	7, 10, 12	13♂ 3♀
STR × C57 black	4, 5, 7, 7½, 8	19♂ 6♀
(C57 black × DBA) × DBA	5, 7, 8, 9	19♂ 1♀

and were used when their parasitemia was about 30 per cent. Heparinized whole blood was sufficiently diluted with physiological saline so that each 0.1 ml. contained 1 million parasitized erythrocytes. Recipient mice were given 0.1 ml. of this inoculum through a vein in the tail.

The recipient mice were of four genetic backgrounds and were bred and weaned at the NIH animal breeding facilities. Two of the groups of mice were F_1 hybrids of inbred strains of mice: STR × C 57 Black and C 57 Leaden × A (LAF). One of the groups of mice was the backcross (C 57 Black × DBA) × DBA; these will be called backcross mice for convenience. The white Swiss mice were not inbred. As seen from Table 1, the mice were mature, varying in age from 2 to 12 months, and all but the white Swiss mice were predominantly males. Though the range in ages was large, there were corresponding groups of mice of each genetic extraction of approximately the same age, again with the exception of the white Swiss.

Blood smears were prepared from tail blood and were stained with Giemsa.

RESULTS

In the first series of experiments, the infection in white Swiss mice was compared in turn with that in LAF, STR × C 57 Black, and backcross mice. As a check on reproducibility, the experiment was repeated comparing STR × C 57 Black and white Swiss mice. In this series of trials the following observations were made daily, with a few lapses, on every mouse: (1) total per cent parasitemia, (2) per cent of mature erythrocytes infected, (3) per cent of immature erythrocytes infected, (4) per cent of all erythrocytes which were immature, and (5) total erythrocytes per cubic millimeter. Time of death of each mouse was recorded.

The observations, in the form of mean daily values for one trial of each kind of mouse, are recorded in Figures 1 through 5. In all the mice the infection in mature erythrocytes reached a peak on or about day 7 and thereafter declined (Figure 1).* It is apparent from this figure that the rate of increase of the infection in mature erythrocytes, as well as the highest density observed, varied with the type of mouse. In white Swiss mice about 50 per cent of the mature erythrocytes were infected; in STR × C 57 Black mice less than 10 per cent were infected.

* This figure and the others in this paper appeared in a review article in the Indian Journal of Malariology and are reproduced by permission of the editor of that Journal.

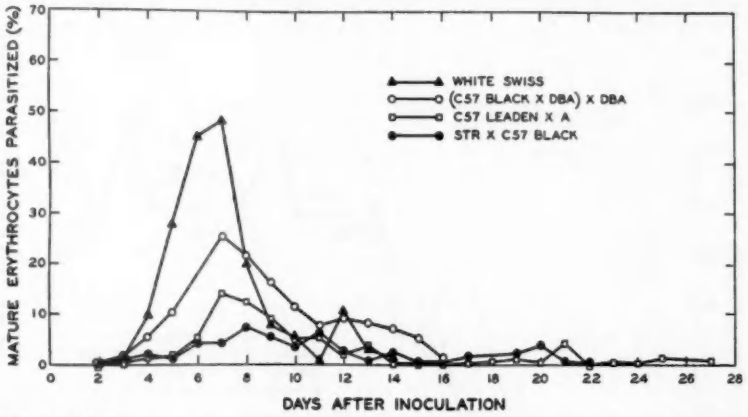

FIG. 1: The course of *P. berghei* infection in mature erythrocytes of four genetically distinct strains of mice.

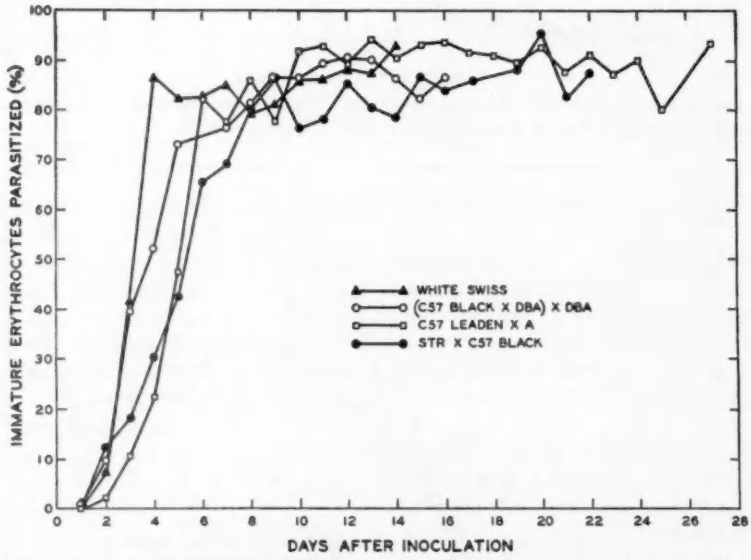

FIG. 2: The course of *P. berghei* infection in immature erythrocytes of four genetically distinct strains of mice.

The infection in immature erythrocytes (Figure 2) rose rapidly in all mice and reached a plateau of between 80 and 90 per cent in 4 to 6 days. There is no indication that in any of the types of mice there was any significant decrease in the per cent infection of immature erythrocytes once a maximum had been reached. The differences among the types of mice during the first week may be real, but

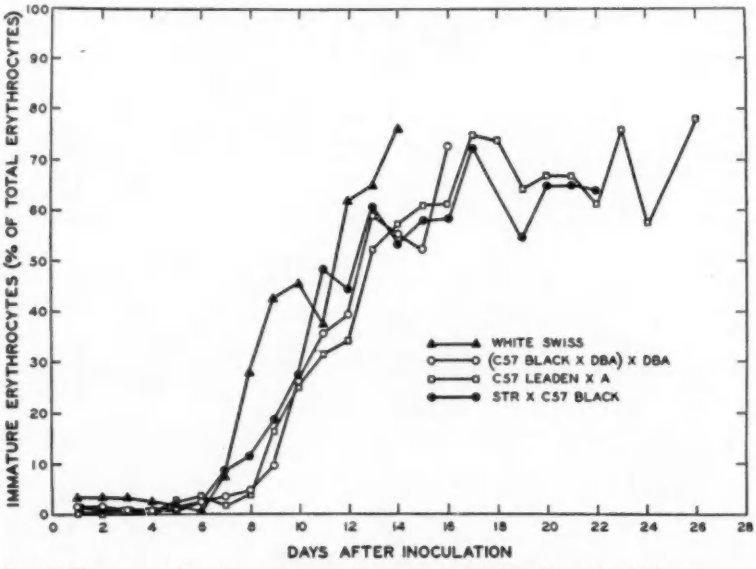
IMMATURE ERYTHROCYTES (% OF TOTAL ERYTHROCYTES)
DAYS AFTER INOCULATION
WHITE SWISS
(C57 BLACK X DBA) X DBA
C57 LEADEN X A
STR X C57 BLACK

FIG. 3: The proportion of immature erythrocytes to total erythrocytes during the course of a *P. berghei* infection in four genetically distinct strains of mice.

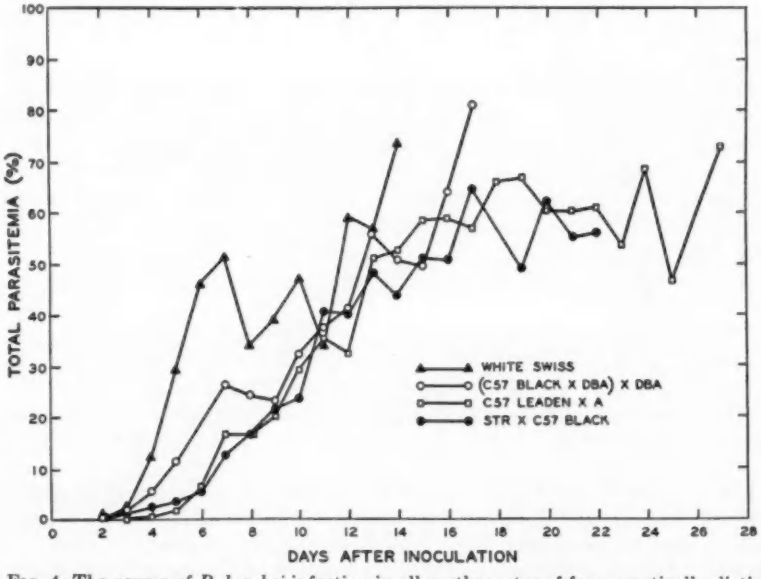

FIG. 4: The course of *P. berghei* infection in all erythrocytes of four genetically distinct strains of mice.

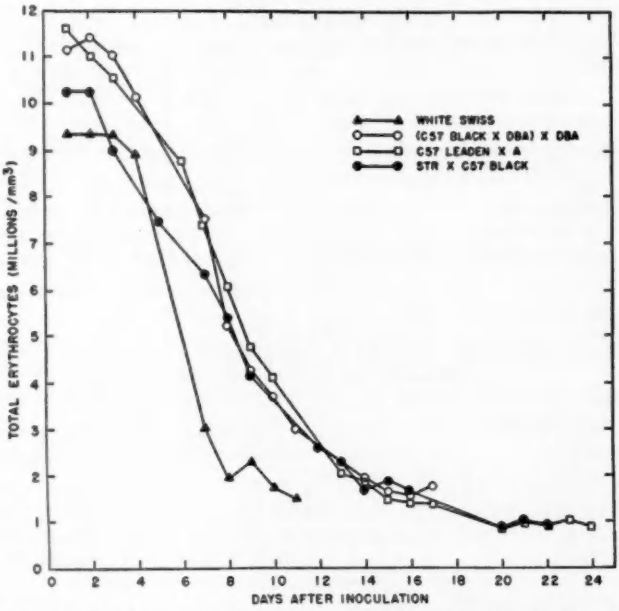

FIG. 5: Total red cell count in four genetically distinct strains of mice infected with *P. berghei*.

they could be attributed to sampling errors with so few immature erythrocytes present in circulation (Figure 3).

During the first week after inoculation, the proportion of erythrocytes which were immature remained low or actually appeared to decline (Figure 3). Thereafter, there was a progressive increase in the proportion of immature erythrocytes until an unstable plateau was reached at the beginning of the second week. The rate of increase in the proportion of immature cells may have been more rapid in the white Swiss mice, but there were probably no significant differences among the other types of mice.

The per cent of all erythrocytes infected (Figure 4) reflects a combination of all the factors described in Figures 1 to 3. The initial peak of infection, distinguishable in some types of mice, can be attributed to the rise and fall in the per cent of mature erythrocytes infected. This initial peak has been described by Schneider and Montézin, 1950; Mercado and Coatney, 1951; Darrow *et al.*, 1952; and by Singer, 1954. The secondary rise in parasitemia can be attributed to the increasing proportion of immature erythrocytes, most of which were infected. In those mice in which there was no decided peak of infection in mature erythrocytes, there was only a vestige of a primary peak of infection.

There is some evidence (Figure 5) that the initial or normal red cell count differs among the types of mice. The erythrocytes per volume began to decline in all mice by the third or fourth day. It dropped more sharply in Swiss mice than

TABLE 2

Mean parasitemia on day seven of four types of mice

Type of mouse	Number of mice*	Mean % infection of mature erythrocytes (day 7)
White swiss	19	50.69 ± 3.99
(C57 black × DBA) × DBA	19	20.83 ± 1.94
C57 leaden × A	16	10.59 ± 1.96
STR × C57 black	24	5.74 ± 1.72

* Differences between these figures and those in Table 1 are mice which failed to survive to day 7.

TABLE 3

Survival of mice in the present studies compared with that of a previous study (Nadel, Greenberg, Jay, and Coatney, 1955)

Type of mouse	Mean survival	
	Present†	Previous‡
White swiss	7.6 ± 0.47	8.4 ± 0.23*
(C57 black × DBA) × DBA	16.55 ± 1.27	13.83 ± 0.48
C57 leaden × A	23.33 ± 1.52	19.29 ± 0.76
STR × C57 black	15.56 ± 0.95	24.45 ± 0.44

* Not previously reported.
† Age given in Table 1.
‡ Age about 2 months ±1 week.

in the others. But among the other types there was a similar rate of decline in erythrocyte numbers.

In the above series of experiments, the most significant factor examined, in terms of the degree of differences and the reliability of the observations, was the course of the infection in mature erythrocytes. Observations on this factor were extended in the following experiments. In this series, representatives of the various types of mice were inoculated at the same time, with the same number of parasites, from the same inoculum. Two observations were made: survival, and the per cent infection of mature erythrocytes. The data from this group of experiments have been pooled with those of the first series and the results of day 7 are shown in Table 2. The contrast in the infection between white Swiss mice and the STR × C 57 Black hybrids is striking; infections in the backcross mice were intermediate between the two extremes. There was a large spread in the data in all groups, and the higher the infection, the larger the spread. Another point of possible significance is the fact that the peak of infection was reached, in general, a day earlier in the Swiss and backcross mice than in the STR × C57 Black and LAF mice. For comparative purposes, the mean infection on day 7 is given in Table 2 for each type of mouse. The difference between the STR × C 57 Black and LAF mice is of no significance ($p < 0.1$), but the differences among all the other types are highly significant ($p < 0.001$).

The survival of all the mice in the present study is given in Table 3. For reference purposes, the survival data for a previous experiment are also given. Most of the latter data have been reported elsewhere (Nadel *et al.*, 1955). All the animals of the previous experiment were approximately two months old. Those in the present series, except the Swiss mice, were 2 to 10 months older. It has been reported (Greenberg *et al.*, 1953) that older mice infected with malaria survive longer than young mice. This could account for the increased survival of the LAF and backcross mice of the present series. The increase in survival in these two types of mice between this and the earlier experiment may be significant ($p < 0.05$). The difference in survival between Swiss mice of the previous experiment and the present one is not significant ($p < 0.2$). The order and the approximate magnitude of the survival data for these three types of mice (Swiss, backcross, and LAF) was approximately the same, considering the age factor, for both series of experiments. On the other hand, the 103 STR × C 57 Black mice which were two months old in the earlier experiments lived significantly longer (9 days, $p < .001$) than the older mice in the present experiment. It is difficult to reconcile this difference with a possible change in the parasite or in technique between the two experiments. It can be tentatively concluded that the rule of increased survival with age is reversed in the STR × C 57 Black hybrid. Such a possibility would be worthy of further investigation.

DISCUSSION

It was pointed out earlier (Nadel *et al.*, 1955) that survival of mice, regardless of genetic background, was bimodal, with one peak of death on day 6 and the other on day 21. It was also observed that a peak of pathological changes, suggestive of acute toxemia, occurred in mice about the end of the first week (Highman *et al.*, 1955). In the present studies it was found that the peak infection of mature erythrocytes occurred on or about the seventh day and that in most animals the maximum decline in red cell count occurred between the sixth and eighth day. The coincidence of these observations: maximum death, peak infection in mature erythrocytes, maximum decline in erythrocyte numbers, and peak pathological changes suggests a causal relationship among these factors. For instance, one could postulate that death at the end of the first week was the result of an acute infection of mature erythrocytes which led to the rapid destruction of mature erythrocytes, and acute toxemia. One would then expect that the higher the peak infection in mature erythrocytes, the more rapid would be the destruction of erythrocytes and the more likely the mouse to die an early death. Such a relationship would appear to be partially true among three of the types of mice used in the present study. The Swiss, backcross, and LAF mice form a consistent series in regard to maximum infection in mature erythrocytes and mean survival. There are, however, certain inconsistencies. The STR × C 57 Black mice are anomalous. On the basis of the rule given above, one would expect them to live as long or longer than the LAF mice. In an earlier experiment with younger mice, they did live significantly longer than the LAF mice. In this experiment (Table 3), they obviously did not. The rule is also of doubtful

validity when one tries to predict the fate of any individual mouse based on the infection of its mature erythrocytes. On the whole, mice with the highest infection of mature erythrocytes died earliest, but those with the lowest infections did not die latest.

But perhaps the rule has the greatest difficulty reconciling the peak infection in mature erythrocytes with the rate of fall in the erythrocyte count. With the exception of the Swiss mice, in which the decline in erythrocyte numbers and the corollary increase in the proportion of immature erythrocytes occurred earlier than in the other mice, the other types of mice were quite similar in these factors.

The coincidence of the factors mentioned above remain, though the explanation of the relationship of the factors is not explained satisfactorily. Evidently, certain data are missing which might tie together death, peak infection of mature erythrocytes, decline in erythrocyte numbers, and pathological changes.

On the other hand, of all the factors studied, infection in mature erythrocytes most clearly differentiates the types of mice studied. This brings up the problem of the preference exhibited by *P. berghei* for immature erythrocytes. This preference has been reported many times (Galliard, 1949; Baldi, 1950; Ramakrishnan and Prakash, 1950; Corradetti and Verolini, 1951; Hsü and Geiman, 1952; Singer, 1953 and 1954). It is apparent from the results of the present experiments that the preference for immature erythrocytes is relative and varies with the strain or type of mouse. It is also apparent that the degree of preference even within a strain varies with the stage of the infection. The preference for immature erythrocytes would be difficult to prove for Swiss mice during the first week of the infection.

It is possible that there is an immunological explanation for the apparent preference of *berghei* for immature erythrocytes; infected mature erythrocytes are removed from circulation more completely and more quickly than infected immature erythrocytes. Certainly, most of those phenomena called immunological have to do with infected mature erythrocytes rather than with infected immature erythrocytes. There is evidence in some types of mice of innate immunity directed toward mature erythrocytes. When compared with the white Swiss mice, the STR × C 57 Black mice would appear to have some mechanism which limits the number of infected mature erythrocytes in the peripheral circulation during the first week of the infection. At the end of the first week there is in all mice which survive long enough, what may be called a crisis, i.e., a rapid destruction, or retention in the deep tissues, of infected mature cells. No comparable events occur in regard to immature erythrocytes; there is no evidence of significant innate immunity and certainly no sign of a crisis. One could assume that the rate of invasion of mature erythrocytes was the same in all types of mice; only the rate of destruction was different. This might account for the fact that the fall in erythrocyte count bears no reasonable relationship to parasitemia. By the first week, mature erythrocytes appear to be removed from the circulation as rapidly in mice with low parasitemia as in those with a high parasitemia. Alternatively, the destruction of mature erythrocytes could be an "all or none" phenomenon, resulting from toxins released by the parasites.

It is obvious that many significant data are missing. The use of different strains of mice and their hybrids has served to point out the gaps in our knowledge of immunity in mice toward malaria. There is evidence which suggests that some of the factors may be genetically controlled. There is also evidence suggesting that mice differ in their ability to survive the acute stress of the infection at the end of the first week. This ability may also be genetically controlled and may be independent of the immunological response of the host to the parasite.

SUMMARY

The following kinds of mice were infected intravenously with *Plasmodium berghei*: white Swiss, C 57 Leaden × A, STR × C 57 Black, (C 57 Black × DBA) × DBA. They were examined daily for total per cent parasitemia; per cent mature erythrocytes infected, per cent immature erythrocytes infected, per cent erythrocytes which were immature, and total red cell count.

There was a peak infection of mature erythrocytes at the beginning of the second week, after which infection in these cells declined to a very low level. The infection in immature erythrocytes rose rapidly in all mice and reached a plateau of about 90 per cent in 4 to 6 days. The proportion of immature erythrocytes to total erythrocytes rose after the first week to a plateau of about 60 per cent. Total parasitemia reflected the initial peak of mature erythrocytes followed by the rise in the proportion of immature erythrocytes, most of which were infected.

The red blood cell count began to drop by the third or fourth day after inoculation. It fell more rapidly in Swiss mice than in the others, but there was little difference among the other mice in this respect.

A more detailed examination was made of the course of the infection in mature erythrocytes. The Swiss mice had a mean count on day seven of 51 per cent; (C 57 Black × DBA) × DBA, 21 per cent; C 57 Leaden × A, 11 per cent; STR × C 57 Black, 6 per cent.

In general, the higher the infection in mature erythrocytes, the shorter lived the mice. There were exceptions and these were discussed. Also discussed was the coincidence of peak infection of mature erythrocytes and the first mode of a bimodal survival curve.

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INTESTINAL PARASITIC INFECTIONS IN FORSYTH COUNTY, NORTH CAROLINA. IV. DOMESTIC ENVIRONMENTAL SANITATION AND THE PREVALENCE OF *ENTAMOEBA HISTOLYTICA*

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Certain of the findings of a survey of the prevalence of intestinal parasitic infections in Forsyth County, North Carolina, have been previously reported (Mackie, T. T. *et al.*, 1955). These relate to the prevalence of *Entamoeba histolytica*, the mass treatment of amebiasis and certain aspects of the epidemiology of the infection. The survey was directed primarily to the examination of 1,934 school children, white and Negro, urban and rural, constituting a representative sample of the schoolchild population of Forsyth County, the examination of family members of children found to be infected by *E. histolytica* and to the examination of a comparable group of negative control families of apparently uninfected children.

A prevalence of 6.1 per cent infection was found among the school children examined. Similar investigation of a total of 94 families of children infected by *E. histolytica* revealed a prevalence of 34.1 per cent, or an average of 2.2 cases of amebiasis per family. Fifty of these families and 49 families of children not found infected at the examinations in the schools were studied to evaluate the usefulness of the examination of school children as an indication of the prevalence of *E. histolytica* in a community. All families utilized in this phase of the investigations were urban residents of the City of Winston-Salem. The observed prevalence of *E. histolytica* among the members of the positive families was 20.7 per cent, more than 30 times greater than that observed among the members of the negative control group, among whom the prevalence was only 0.6 per cent.

Although the two groups of families were comparable in all respects which could be controlled, the observed prevalence was almost twice as great in the Negro families as in the whites. Since no special predilection to amebiasis among Negroes has been demonstrated, it appeared probable that the observed difference might be attributable to greater exposure to infection due to a poorer sanitary environment. The results of the investigations of the domestic sanitary environment are the subject of this report.

EPIDEMIOLOGY

Polluted water, the use of night soil as a fertilizer, mechanical spread by flies and contamination of food by infected food handlers are accepted factors in the epidemiology of amebiasis. Until comparatively recently, *E. histolytica* has not been actually demonstrated except in association with flies, man and certain potential reservoir hosts. Since man is both the predominant reservoir and the

ultimate host, the local sanitary environment must determine prevalence. It has been rightly said by Connel and French (1939) that "amebic infection depends more on how one lives than where one lives."

Although water undoubtedly plays a part in the spread of infection in regions where there is gross contamination of streams and shallow wells with human feces, it is improbable that it is a usual means of transmission. The water-borne infections that have been investigated in detail characteristically tend to be explosive in nature and to be accompanied by considerable numbers of cases of diarrhea and dysentery. Furthermore, comparison of the prevalences of *E. histolytica* among families using polluted and unpolluted local water supplies has shown little difference in the infection rate (Eyles *et al.*, 1953).

Since Wenyon and O'Connor (1917) first showed that cysts of *E. histolytica* may be recovered from the intestinal tract of flies up to seventy-two hours after an experimental feed on infected human excreta, the potential importance of this insect as a vector of amebiasis has been definitely established. Using development in suitable culture media as the criterion, viable cysts have been recovered from the vomitus (Pipkin, 1942) and from the droppings of flies up to 24 hours after an experimental feed on infected material (Sieyro, 1942). Furthermore, cysts recovered from flies caught near latrines and in a house have been proved to be *E. histolytica* both by culture and subculture and by Heidenhain-stained preparations of the fly droppings (Harris and Down, 1946).

Years ago Craig (1934) laid down the dictum that "the contamination of food and drink by food-handlers who are passing cysts of *Endamoeba histolytica*, the so-called 'carriers', is undoubtedly the most common and most important method of transmission in many localities. . . ." Although this has been a somewhat controversial topic, convincing evidence of the reality of the threat of the infected food handler has been presented by Schoenleber (1940). The prevalence of amebiasis among foreign personnel on Aruba was found to be 25 per cent and among the food handlers 33 per cent. Improvements in hygiene, treatment of infected persons and refusal of employment as food handlers to infected individuals, were followed within one year by a reduction of 50 per cent and after three years by a reduction of 92 per cent in the infection rate. The importance of this method of transmission is further supported by the report that cysts may be recovered from hand-washings and nail scrapings from 6 per cent of infected persons immediately after defecation (Spector *et al.*, 1935).

Recently, experimental and epidemiologic evidence has been advanced in support of the possibility of transmission by contact with contaminated objects other than food or drink. In the course of an investigation into the reasons for the persisting high endemicity of amebiasis in a New Orleans orphanage by Ivanhoe (1943), special techniques revealed possible similarities to the epidemiology of enterobiasis. Examination of centrifuged washings and of NIH swabs taken from many areas in the institution demonstrated the presence of cysts morphologically corresponding to the cysts of *E. histolytica*. These were recovered from such diverse sources as the hands and soiled underwear of the children, the bottom of the laundry chute, damp sand in a playbox in the yard,

the contents of the wading pool and the bottom of the pool after it had been drained. Further epidemiological evidence was obtained in the course of two studies of rural communities in Tennessee by Eyles *et al.* (1953, 1954). The findings seemed to be incompatible with the hypothesis that transmission is confined to food handlers, flies and water. A direct correlation was shown between the prevalence of *E. histolytica* the methods of excreta disposal and the general cleanliness of the home. Similarly, infections were found to be more numerous among families using unsanitary privies than among others having no toilet facilities. In both investigations the observed conditions of prevalence could best be explained by infection resulting from contact with infected persons or with articles contaminated by infected persons.

POPULATION SAMPLE

Two population samples were used for the investigation of a possible relationship between the domestic sanitary environment and the observed prevalence of *E. histolytica*. The first included 1,115 of the 1,934 children examined in the schools. They constitute a representative sample of the white and Negro urban school population of the middle and lower socio-economic strata of Forsyth County. These students do not represent 1,115 different families. Although it was possible to ascertain siblings in the positive families (at least one member infected by *E. histolytica*) and in the negative control families (non-infected) used in the studies of familial amebiasis, it was not feasible for the negative non-control students. However, it was considered probable that the number of siblings in school was comparable in all three groups, hence the comparisons made are not seriously biased by counting siblings more than once. The children comprising this first group fall into three categories: 934 who were not found to be infected by *E. histolytica* at the examinations in the schools and who were not utilized in the other studies; 70 children not found to be infected but whose family members were used as the control group for the evaluation of the case-finding technique; and 111 students found infected by *E. histolytica* in the school survey who were used as probands to the positive families for the studies of familial amebiasis.

The second group comprises 849 family members, including the children who served as probands in the studies of familial amebiasis. It includes 230 members of the urban negative control families, 360 members of the urban positive families and 259 members of the rural positive families.

METHODS

An objective inventory schedule was prepared by the Department of Sanitary Engineering, School of Public Health, University of North Carolina. The individual sanitation facilities were tabulated under three major headings: water supply, sewage disposal and garbage disposal. Each facility was classified into mutually exclusive types. Recording was done by checking the appropriate description of the particular facility. Application of an elimination screen was utilized to establish a numerical grade for the overall facilities of each family.

This yielded four well-defined grades of domestic environmental sanitation based on factual data which eliminated the bias of interpretation and judgment of a sanitary inspector. The simplicity of this method and the preponderance of objective data in the determination of the sanitation grade made it preferable to the alternate procedure of allotting points for each facility. Furthermore the latter method tended to give a less accurate estimate of the true conditions of environmental sanitation if insufficient weight were given to undesirable features.

For assignment to one of the three higher grades, a dwelling had to have all the items listed for that grade. All residences which failed to meet the specifications for one of the first three grades were automatically classified as Grade IV, below minimum sanitation facilities. If any one item was lacking, the residence was classified in one of the lower grades so that, in effect, the actual sanitation evaluation would classify the dwelling in the grade that included the poorest facility found. This method was recognized as particularly severe for the rating of dwellings dependent upon rural types of facilities.

Grade I indicates optimal conditions of sanitation. It required connection to the city water system, private inside flush toilet connected to the city sewage system, and regular garbage removal by the city or by a private collector.

Grade II indicates the presence of all essential but not the optimal facilities. The criteria for this grade comprised connection to the city water supply or a private well equipped with pump and pressure service to the house, private inside flush toilet or inside flush toilet shared with another family but connected to the city sewer or to a septic tank, and regular removal of garbage by the city or by a private collector.

Grade III indicates minimal acceptable facilities. These consisted of connection to the city water system or city water obtained from a neighbor's tap or from an outlet in the yard, inside flush toilet shared with others but connected to the city sewer system or to a septic tank, or an outside privy with concrete floor for the use of the family only, and family handling their own garbage disposal.

Grade IV indicates environmental sanitation below minimum standards. This classification included water supply from private well equipped with hand pump or private well without pump, flush toilet in yard shared with other families or outside privy shared with other families, or privy unit with wood or earth floor, and no suitable garbage disposal.

The Division of Engineering of the City-County Health Department, Forsyth County, offered their assistance in this phase of the survey and generously made the sanitation evaluations of the homes of all students examined in the urban schools. The evaluations of the rural homes were made by a member of the survey team. These were restricted to the dwellings of children found infected by *E. histolytica*, since evaluations of the residences of all the rural families covered by the survey proved too large an undertaking to be practicable.

FINDINGS

Water supply. The data pertaining to the water supply of the individual homes are presented in Table 1. Of the consolidated group of 1,115 school children, the

TABLE 1

Type water supply and prevalence of Entamoeba histolytica *among urban school children*

Type*	Negative non-control		Negative control		Positive families		All groups		
	Total	Number positive	Total	Number positive	Total	Number positive	Total	Number positive	Per cent infected
a	703	0	56	0	68	43	827	43	5.2
b	69	0	4	0	33	26	106	26	24.5
c	0	0	0	0	0	0	0	0	0.0
d	0	0	0	0	1	1	1	1	100.0
e	0	0	0	0	0	0	0	0	0.0
Unknown	162	0	10	0	9	5	181	5	2.7
Totals	934	0	70	0	111	75	1,115	75	6.7

* a—connection to city water supply; b—city water from neighbor's tap or yard tap; c—private well with pressure service piped into house; d—private well with hand pump; e—private well without pump.

domestic water supply for 827 was obtained directly from the city distribution system through a private outlet; 106 depended upon a neighbor's tap or an outlet in the yard of the house; one used a shallow well equipped with hand pump; and the source of water for 181 was not determined. The overall prevalence of *E. histolytica* in this group of 1,115 children was 6.7 per cent.

Comparisons of the prevalences in the subgroups using the different types of installation indicate a definite and significant correlation of infection with the type of domestic water supply. Infections were found in only 5.2 per cent of those who obtained their water directly from the city distribution system through a private outlet. In contrast to this, infections were demonstrated in 24.5 per cent of those using a neighbor's faucet or an outdoor outlet in the yard. The one child whose family depended upon a shallow well was among those found to be infected.

The positive family group of 111 children included 75 in whom infections by *E. histolytica* were demonstrated. Sixty-eight obtained their water directly from the city supply system and 33 used a neighbor's outlet or an outside tap in the yard. The infection rate among the latter was 78.8 per cent.

Excreta disposal. Evaluation of the sanitary environment of the individual homes in relation to excreta disposal was based first on the type of toilet facility available to the family and secondly on the method of disposal. The data relating to the type of installation are shown in Table 2.

The significance of the type of toilet facility available to the family becomes apparent when the prevalence of 6.7 per cent infection in the consolidated group of 1,115 school children is compared with the prevalences in the subgroups using different types of facilities. Apart from those whose type of installation was undetermined, the lowest observed prevalence of *E. histolytica*, 5.7 per cent, was found among those who had private inside flush toilets. If a flush toilet was available within the house or in the yard but was shared with other families, the observed prevalence was approximately twice as great, 11.5 and 11.6 per cent,

TABLE 2

Excreta disposal (type of installation) and prevalence of Entamoeba histolytica *among urban school children*

Type*	Negative non-control		Negative control		Positive families		All groups		
	Total	Number positive	Total	Number positive	Total	Number positive	Total	Number positive	Per cent infected
1	622	0	45	0	69	42	736	42	5.7
2	62	0	14	0	11	10	87	10	11.5
3	79	0	1	0	15	11	95	11	11.6
4	5	0	0	0	7	7	12	7	58.3
5	3	0	0	0	0	0	3	0	0.0
Unknown	163	0	10	0	9	5	182	5	2.7
Totals	934	0	70	0	111	75	1,115	75	6.7

* 1—Inside flush toilet; 2—Inside flush toilet shared with other families; 3—Flush toilet in the yard shared with other families; 4—Private privy for the family only; 5—Privy shared with other families.

TABLE 3

Excreta disposal (method) and prevalence of Entamoeba histolytica *among urban school children*

Method*	Negative non-control		Negative control		Positive families		All groups		
	Total	Number positive	Total	Number positive	Total	Number positive	Total	Number positive	Per cent infected
1	766	0	60	0	95	63	921	63	6.8
2	1	0	0	0	0	0	1	0	0.0
3	0	0	0	0	4	4	4	4	100.0
4	4	0	0	0	3	3	7	3	42.9
5	0	0	0	0	0	0	0	0	0.0
Unknown	163	0	10	0	9	5	182	5	2.7
Totals	934	0	70	0	111	75	1,115	75	6.7

* 1—Flush toilet connected to city sewer; 2—Flush toilet connected to septic tank; 3—Privy with concrete floor; 4—Privy with wood or earth floor; 5—Privy adequately fly-proofed.

respectively. Among those having outside privies for the exclusive use of the family, the prevalence was 58.3 per cent, five times greater than among those using shared flush toilets and ten times greater than among those having private flush toilets.

In contrast to the two negative groups, almost one-third of the children comprising the infected family members had toilet facilities falling into categories 2, 3 and 4.

These observations suggest that there is an increased hazard of direct contamination in shared toilets and privies and a greater probability of fly transmission of amebae, particularly in the latter type facility.

TABLE 4

Garbage disposal methods and prevalence of Entamoeba histolytica *among urban school children*

Method*	Negative non-control		Negative control		Positive families		All groups		
	Total	Number positive	Total	Number positive	Total	Number positive	Total	Number positive	Per cent infected
a	766	0	60	0	97	66	923	66	7.1
b	0	0	0	0	0	0	0	0	0.0
c	5	0	0	0	5	4	10	4	40.0
Unknown	163	0	10	0	9	5	182	5	2.7
Totals	934	0	70	0	111	75	1,115	75	6.7

* a—Garbage collected by the City; b—Garbage removed by private collector; c—Family disposes of own garbage.

The method of excreta disposal also indicates an important relationship to the prevalence of *E. histolytica*, Table 3. The prevalence of *E. histolytica* among the 921 persons who used flush toilets connected to the city sewage system was 6.8 per cent, approximating the 6.7 per cent for the entire group of 1,115 children. The striking fact here is the high prevalence observed among the few persons whose toilet facilities consisted of a privy, irrespective of the type of construction. The difference noted between the concrete-floored privy and wood or earth-floored is not statistically significant. Correlating with this is the fact that no adequately fly-proofed privies were found. This constitutes further indirect evidence of the possible importance of fly transmission of the infection.

Although the type of sewage disposal available for 182 of the total group was undetermined, the prevalence of *E. histolytica* of 2.7 per cent, would indicate that the majority of the installations fell into the top sanitary brackets.

Garbage disposal. Investigation of the methods of garbage disposal yielded results which correlate well with the observations discussed above and provides further indirect evidence of the potential importance of fly transmission. The majority of the people had available the city garbage collection service. Among these persons, the prevalence of *E. histolytica* was 7.1 per cent. Among 182 individuals for whom the method of garbage disposal was not ascertained, the prevalence was 2.7 per cent. The data are shown in Table 4.

In the small group of 10 persons who disposed of their own garbage, the prevalence was 40.0 per cent. This difference is of striking magnitude and, despite the smallness of the group, it is statistically significant. The direction of the difference points to the recognized high rate of transmission when fly breeding is uncontrolled and there is an immediate source of contamination by human excreta in the environment.

Grade of sanitary facilities. It has been stated previously that the sanitary data for each family relating to water supply, excreta disposal and garbage disposal were subjected to an elimination screen to derive the final sanitary grade. In Table 5 the 1,115 student members of the urban families whose environmental

TABLE 5

Grade of sanitary facilities and prevalence of Entamoeba histolytica *among urban school children*

Grade*	Negative non-control		Negative control		Positive families		All groups		
	Total	Number positive	Total	Number positive	Total	Number positive	Total	Number positive	Per cent infected
I	617	0	45	0	61	37	723	37	5.1
II	50	0	11	0	4	4	65	4	6.2
Totals	667	0	56	0	65	41	788	41	5.2
III	18	0	3	0	17	13	38	13	34.2
IV	86	0	1	0	20	16	107	16	14.9
Totals	104	0	4	0	37	29	145	29	20.0
Unknown	163	0	10	0	9	5	182	5	2.7
Grand totals	934	0	70	0	111	75	1,115	75	6.7

* I—Optimal conditions of sanitation; II—Essential but not optimal facilities; III—Minimal acceptable facilities; IV—Below minimum standards of sanitation.

sanitation was investigated are grouped in accordance with the final sanitary grade. It will be recalled that Grade I indicates optimal conditions; Grade II, essential facilities but not optimal; Grade III, minimal acceptable sanitary facilities; and Grade IV, below minimum standards of sanitation.

Comparison of the three groups of children investigated reveals some significant differences in the proportions of each group falling into the four grades. Slightly less than one-sixth of the negative non-control family members were living in Grade III or Grade IV dwellings. Only one-seventeenth of the negative control family members were living under these conditions. In contrast to these groups, one-third of the positive family members resided in dwellings classified as Grade III or Grade IV.

The correlation between the prevalence of infection and the sanitary rating is underscored when the three groups are consolidated. The prevalence among 788 children living under Grade I or Grade II conditions was 5.2 per cent. Among the 148 living under Grade III or Grade IV conditions, it was 20.0 per cent.

The preceding discussion has been limited to *E. histolytica* prevalence in relation to the sanitary grades of the domiciles of urban families only. Similar grading of the environmental sanitation of the infected rural families was done by members of the survey team. It was not possible to extend this to include rural families of children not found infected in the survey. Comparison of all the family members who were examined in the three groups, i.e., the urban negative controls, the urban positive family members and the rural positive family members, reveals the same correlation between prevalence of *E. histolytica* and the grades of the sanitary facilities. The data are presented in Table 6.

In Table 6, the 230 urban negative control family members are contrasted

TABLE 6

Grade of sanitary facilities and prevalence of Entamoeba histolytica *among negative control, urban and rural positive families*

Grade	Urban negative families			Urban positive families			Rural positive families			Urban and rural positive families		
	Total	Positive		Total	Positive		Total	Positive		Total	Positive	
		No.	%		No.	%		No.	%		No.	%
I	148	0		178	59		16	3		194	62	
II	39	1		15	5		4	1		19	6	
Totals	187	1	0.5	193	64	33.2	20	4	20.0	213	68	32.0
III	8	0		59	22		30	9		89	31	
IV	3	0		77	30		209	75		286	105	
Totals	11	0	0.0	136	52	38.2	239	84	35.1	375	136	36.2
Unknown	32	0		31	7		0	0		31	7	22.6
Grand totals	230	1	0.4	360	123	34.2	259	88	34.0	619	211	34.1

TABLE 7

Grade of sanitary facilities and prevalence of E. histolytica *among the negative control and urban and rural positive families*

Sanitary grade	Combined family groups		
	Total individuals	Number positive	Per cent infected
I	342	62	18.1
II	58	7	12.1
Totals	400	69	17.3
III	97	31	32.0
IV	289	105	36.3
Totals	386	136	35.2
Unknown	63	7	11.1
Grand totals	849	212	25.0

with 619 positive family members comprising the urban and rural family groups examined. Only one infection by *E. histolytica* was found among the controls, a prevalence of 0.4 per cent. The consolidated groups of positive family members had 211 infections, a prevalence of 34.1 per cent. There is no difference in prevalence between the urban and the rural positive family members, 34.2 per cent and 34.0 per cent, respectively.

The observations demonstrate that, in both the urban and the rural groups, there is a significantly higher prevalence of infection by *E. histolytica* among family members living under sanitary conditions classed as minimum acceptable or below minimum standards. Among the 329 positive urban family members

with known sanitary grade, 193 or 58.7 per cent were living under sanitary conditions which were graded I or II. In contrast, the urban negative families had 94.4 per cent of their members in these two grades of better sanitary facilities. This difference is highly significant and is evidence that a direct causal relationship exists between the quality of sanitary facilities and infection by *E. histolytica*.

Only 20 of the 259, or 7.7 per cent of the rural positive family members, were classified in the sanitary Grades of I and II. The difference between urban and rural, viz. 58.7 versus 7.7 per cent, can be ascribed to the sanitary rating scheme itself. Although both groups were positive, the rating scheme was designed primarily to meet the needs of an urban group and tends to penalize rural facilities. Therefore, the fact that the rural group was positive explains why there was so negligible a percentage with Grade I and II facilities.

Another method of establishing the relationship between the quality of the sanitary facilities and the prevalence of infection is to examine the prevalence rates in the consolidated groups, Table 7. As the sanitary facilities decline in quality, the prevalences increase as follows: 18.1, 12.1, 32.0, 36.3 per cent. The difference between Grades I and II is probably due to chance; but a definite trend is seen in progressing from Grade I to Grade IV. The prevalence for Grades I and II is one-half as great as that for Grades III and IV combined. From these results, it can be shown that the risk of infection by *E. histolytica* is almost doubled when a family has a sanitary Grade of III or IV rather than sanitary facilities with the Grade of I or II. This relative increase was estimated by adjustment of the relative frequencies in Table 7, according to the method of Cornfield (1951).

CONCLUSIONS

1. The observed prevalence of *Entamoeba histolytica* bears a definite and significant relationship to the type of domestic water supply, toilet facility, sewage disposal and garbage disposal.

2. Similarly, the application of an elimination screen to permit classification of the individual facilities demonstrates that, as the sanitary grade declines in quality, the prevalence of infection rises.

3. The risk of infection for families living under minimal acceptable or below minimum standards of sanitation is almost double that of families having optimal or essential sanitary facilities.

4. The observations made in the course of this investigation suggest that direct contamination, contact with contaminated objects and fly transmission were all factors in the local epidemiology of amebiasis.

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INTESTINAL PARASITIC INFECTIONS IN FORSYTH COUNTY, NORTH CAROLINA. V. PREVALENCES OF INDIVIDUAL PARASITES

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Previous reports have presented different aspects of the findings of a survey of intestinal parasitic infections conducted in Forsyth County, North Carolina. These investigations were undertaken because of the paucity of general information relating to the area under consideration and because of discrepancies between the observed prevalence of *Entamoeba histolytica* among patients at the North Carolina Baptist Hospital in Winston-Salem and the prevalence of amebiasis as indicated by the morbidity data of the State Department of Health. Since it was necessary to offer treatment to infected persons in order to obtain maximum cooperation in the survey, ambulatory treatment by diiodohydroxyquin (Diodoquin) was made available to all individuals found infected by *E. histolytica*. The primary group examined, children in elementary schools, was utilized for a detailed study of familial amebic infections and to evaluate the usefulness of the examination of school children as a case-finding technique. The examination of urban family members revealed an unexplained higher prevalence of *E. histolytica* among the Negro than among the white families. Since there is no special racial predeliction to amebic infection, it appeared that differences in the family sanitary environment might be responsible (Mackie, T. T. *et al.*: I, II, III, IV).

Previously reported surveys in North Carolina have been almost entirely restricted to determinations of the prevalences of hookworm and of *Ascaris lumbricoides*. During the period 1910–1914, the investigations of the Rockefeller Sanitary Commission revealed a prevalence of hookworm of 29.6 per cent among a total of 278, 664 persons examined. The observed prevalence in Forsyth County was 23.8 per cent (Rockefeller Sanitary Commission, 1915). Surveys conducted prior to 1921 showed a prevalence of infection by *A. lumbricoides* of 5.0 per cent among 36,435 persons from sixteen widely scattered counties (Otto and Cort, 1934).

An extensive re-survey of the prevalence of hookworm infection in the State was published by Keller *et al.* in 1937. This showed that a marked drop in prevalence had occurred in the period since 1910–1914. The rate for 70 counties in the State was reduced to 12.3 per cent among 37,391 persons examined, and in Forsyth County to 2.9 per cent.

This marked decrease in hookworm infection was attributed to a variety of

[1] We gratefully acknowledge the assistance of Mrs. Allen Holderman.

factors. At the time of the initial investigations, existing sanitary conditions left much to be desired. Only 6 of 238 schools for white children inspected were equipped with sanitary privies; 20 Negro schools examined had none; and 14 mills employing 5,700 persons had no sanitary facilities whatever. A state-wide sanitary privy law was enacted in 1919. Enforcement of this law was more effective in the mill towns of the Piedmont area than in other sections of the State and relatively few privies had been installed in rural areas. Consolidation of rural schools with provision of adequate sanitary facilities which afforded protection to school children through a considerable portion of the year had been undertaken some fifteen years previously. These factors were supported and supplemented by education of the public concerning the danger of soil pollution, the continuing interest of physicians in the treatment of hookworm disease, and the establishment of full-time County Health Departments.

Although the presence of intestinal protozoa was noted when observed by the investigators of the Rockefeller Sanitary Commission, the data were incomplete and adequate information concerning the prevalence of these organisms has been lacking.

Population sample. Forsyth County, situated in the Piedmont region of the State, has an area of approximately 409 square miles. The 1950 census reported a total population of 146,135, divided by race and residence as follows:

	Urban	*Rural*
White	51,051	53,642
Negro	36,760	4,682
Total	87,811	58,324

In the statistical planning of the survey it was recognized that examination of a broad sample of the general population would be required to obtain an unbiased estimate of prevalence. The difficulties inherent in such an operation, however, could not be overcome. Decision was made, therefore, to investigate the prevalence of parasitic infections among the school population rather than among the general population. It was believed that the prevalence of these infections would be somewhat higher among the school children than in the adult population. From the former values, however, it would be possible to estimate the prevalence rate in the general population, although bias might be introduced because families without a child in school would not be represented.

Preliminary calculations were made to determine the minimum sample size that would be adequate to show significant differences between the racial and urban-rural groups. A forecast of maximum expected rates of prevalence was used to assure the requirement of a maximum number of school children in the sample. On the assumption that the survey would demonstrate a prevalence of approximately 15 per cent for *E. histolytica*, the selection of 165 children in each category would give a fifty-fifty probability of showing a difference of 5 per cent in opposite directions to be statistically significant at the 1 per cent level. However, calculations indicated that twice this number of children would be required to provide a sufficient number of infections by *E. histolytica* for the treatment studies. As a final precaution, this number was increased by 10 per cent to offset

anticipated lack of cooperation and other unforeseen problems which might affect the validity of the population sample. The final number of school children examined was, therefore, somewhat larger than actually required for the statistical purposes of the study.

Grades II, III, IV and V of the school population were selected for examination. Grade I was omitted because the children were considered too young to give optimal cooperation, they were available in school only for half-day periods, and it was impossible to forecast accurately the number entering school the following year. Grade VI and above were omitted since many students from poor socio-economic backgrounds may not go beyond Grade V and, further, it was considered that these older children would prove less cooperative.

The total school population in Forsyth County by grade, race and residence, whether urban or rural, including the samples used for the survey are shown in Table 1.

The four grades selected provided a total of 10,698 children. Of these, 2,063, approximately one-fifth, comprised the population sample and 1,934 were examined. The individual schools in which the survey was conducted were selected to give proper distribution of the sample by race and residence and to represent the middle and lower economic brackets of the population. The sequence in which the six schools selected were studied was determined by chance.

Children found infected by *E. histolytica* at the examinations in the schools served as probands or leads to their families, which were used for the investigations of familial prevalence of infection and for the evaluation of therapy. This group of 94 urban and rural positive families comprised 449 persons. Similarly a group of apparently uninfected children served as probands to the 49 urban negative control families, 160 of whose members were also examined. Children previously examined in the schools are not included in these totals of family

TABLE 1

School population in Forsyth County during 1950–1951, by race and school location, whether urban or rural

Race and location	Number		Grade II		Grade III		Grade IV		Grade V	
	Available	Sample	A*	S**	A*	S**	A*	S**	A*	S**
Grand total	10,698	2,063	2,961	511	2,806	514	2,521	457	2,410	581
Rural white	4,451	481	1,236	119	1,155	141	1,033	128	1,027	93
Rural Negro	494	447	143	130	124	115	103	94	124	108
Urban white	3,134	590	886	160	834	155	769	137	645	138
Urban Negro	2,619	545	696	102	693	103	616	98	614	242
Total white	7,585	1,071	2,122	279	1,989	296	1,802	265	1,672	231
Total Negro	3,113	992	839	232	817	218	719	192	738	350
Total urban	5,753	1,135	1,582	262	1,527	258	1,385	235	1,259	380
Total rural	4,945	928	1,379	249	1,279	256	1,136	222	1,151	201

* A—number available

** S—number used as sample in survey

members. For the purposes of the survey, a family was defined to include all individuals residing under the same roof and taking at least one meal together daily.

The schoolchild sample included 1,071 whites and 992 Negroes; 55 per cent were urban residents. The 449 positive family members included 183 whites and 266 Negroes; 55 per cent were urban residents. Among the 160 negative control family members, 53 were whites and 107 were Negroes; all were urban residents. The total population sample examined in the course of the survey, therefore, was 2,543 persons.

Cooperation. The cooperation rates represented by these individual population samples were as follows: school children 93.7 per cent; positive family members 90.8 per cent; and negative control family members 86.1 per cent. The children previously examined in the schools are included in the respective totals for the calculation of the cooperation rate in the two family groups. The cooperation rate represented by the entire group of 2,543 persons examined was 91.7 per cent.

Procedures. The procedures involved in the survey were explained to the principals of the schools and to the respective Parent-Teacher Associations. A temporary field laboratory was set up in each school and the examinations were conducted room by room after receipt of signed permission slips from the parents. On the day of examination, immediately on arrival at school in the morning, each child was given crackers and a phenolphthalein-chocolate laxative.[2] Previous field experience had proved this to be the laxative of choice because of its familiarity to the public, its acceptability to children and adults alike, its dependability in producing specimens within the limited time available, and its complete freedom from toxicity and undesirable side effects. Freshly passed fecal specimens were examined immediately after collection, using saline and iodine films and Quensel's-stained preparations. Warm stages were used throughout. At the end of the school day the specimens were taken to the base laboratory for further examination after concentration, using the zinc sulphate centrifugal flotation technique (Faust *et al.*, 1939). The identification of individual parasites was invariably confirmed by at least two members of the survey team and, in the case of doubtful specimens, permanent-fixed preparations were made for subsequent examination after staining by the iron hematoxylin method.

The methods of examination for the family members differed only in that the time lag between evacuation and examination of specimens was somewhat longer. Persons to be examined were instructed to take the laxative agent at night and to collect a specimen the following morning. These were picked up by messenger service and taken to the base laboratory as promptly as possible.

The efficiency of the laboratory diagnostic methods used throughout the survey was calculated using the method of Mantel (1951). Since examination of successive stool specimens was not practicable, calculations were based upon the findings at the pre- and post-treatment examinations of the individuals who received placebo treatment. These indicated a probability of 59 per cent of demonstrating

[2] This preparation was generously supplied by Ex-Lax, Inc.

the *E. histolytica* in a specimen from an infected person by examination of one stool. This compares favorably with the value of 62 per cent efficiency reported by Tobie *et al.* (1951).

FINDINGS

Among the total group of 2,543 persons examined, one or more intestinal parasites were found in 1,319, an overall prevalence of 51.9 per cent.

The 1,934 school children included 1,010 infected individuals, a prevalence of 52.2 per cent; the 449 positive family members included 270 infected persons, a prevalence of 60.1 per cent. In contrast to these figures, only 24.4 per cent of the negative control family members were found to be infected. It is highly significant that the prevalence of infection among the family members of children found not to be infected by *E. histolytica* was less than half the prevalence found in the entire group of 2,543 persons examined.

TABLE 2

Prevalence of intestinal parasitic infections

Group	Number examined	Number positive	Per cent parasitized
School children	1,934	1,010	52.2
Positive families	449	270	60.1
Negative controls	160	39	24.4
Totals	2,543	1,319	51.9

TABLE 3

Prevalence of intestinal parasitic infections in Forsyth County, North Carolina

Parasites	1,934 School children		449 Members Positive families		160 Members Negative controls	
	Number positive	Per cent positive	Number positive	Per cent positive	Number positive	Per cent positive
Pathogenic Protozoa						
E. histolytica	118	6.1	102	22.7	1	0.6
D. fragilis	321	16.6	100	22.3	10	6.3
G. lamblia	159	8.2	46	10.2	9	5.6
Commensal Protozoa						
E. coli	474	24.5	130	29.0	15	9.4
E. nana	391	20.2	137	30.5	8	7.5
I. bütschlii	13	0.7	8	1.8	0	0.0
C. mesnili	15	0.8	6	1.3	1	0.6
T. hominis	5	0.3	2	0.4	0	0.0
Unidentified amebae	11	0.6	5	1.1	0	0.0
Helminths						
Hookworm	15	0.8	5	1.1	0	0.0
S. stercoralis	6	0.3	0	0.0	0	0.0
A. lumbricoides	17	0.9	10	2.2	1	0.6
T. trichiura	7	0.4	0	0.0	0	0.0
E. vermicularis	156	8.1	9	2.0	0	0.0

The prevalences of the more important protozoa and helminths in the three groups are shown in Table 3.

Although the three groups are not directly comparable, the differences in prevalence are of interest. Among the school children the observed prevalences of the pathogenic protozoa were: *Entamoeba histolytica*, 6.1 per cent; *Dientamoeba fragilis*, 16.6 per cent and *Giardia lamblia*, 8.2 per cent. Without exception, infections by these three organisms were encountered more frequently among the positive family members than in the children or the negative control family members. However, the most striking differences lie between the two family groups. Although these are not directly comparable since the negative controls comprised urban residents only, when the comparison is restricted to urban residents of the positive families the observed differences are in the same direction and of the same magnitude.

Only one infection by *Balantidium coli* was found. This was in a nine-year old Negro boy whose home had a high sanitary rating.

Entamoeba coli and *Endolimax nana* were by far the most frequently observed organisms among the non-pathogenic protozoa. The prevalences of these two amebae were two to three times greater among the school children and the members of the positive families than among the negative controls. Infections by the other commensal protozoa were uncommon.

With the exception of *Enterobius vermicularis*, helminth infections were rare. Only 20 instances of hookworm infection were found among the 2,543 individuals examined. Eggs of *Ascaris lumbricoides* were found in 28 of those examined.

TABLE 4

Percentage prevalence of parasitic organisms by race and residence among 1,934 school children and 449 positive family members

Parasites	1,934 School children				449 Positive family members			
	Rural white	Rural Negro	Urban white	Urban Negro	Rural white	Rural Negro	Urban white	Urban Negro
Number of persons	404	425	566	539	67	129	116	137
Pathogenic Protozoa								
E. histolytica	4.9	6.3	6.0	6.9	27.0	23.3	17.2	24.8
D. fragilis	14.5	19.0	19.6	13.4	16.4	19.4	22.4	27.0
G. lamblia	7.6	5.1	10.0	9.2	7.5	7.0	16.4	9.5
Commensal Protozoa								
E. coli	21.6	30.9	19.2	27.3	38.8	38.8	19.8	22.6
E. nana	21.6	17.3	18.9	22.8	23.9	27.1	32.8	35.0
I. bütschlii	1.0	0.5	0.0	1.0	9.0	1.6	0.0	0.0
C. mesnili	0.5	1.0	0.6	1.1	0.0	3.1	0.9	0.7
T. hominis	0.5	0.2	0.0	0.4	0.0	0.8	0.0	0.7
Helminths								
Hookworm	2.9	0.2	0.4	0.0	6.0	0.0	0.9	0.0
S. stercoralis	0.5	0.0	0.7	0.0	0.0	0.0	0.0	0.0
A. lumbricoides	1.2	1.7	0.9	0.0	10.4	1.6	0.9	0.0
T. trichiura	0.7	0.2	0.4	0.2	0.0	0.0	0.0	0.0
E. vermicularis	16.2	4.1	11.4	1.6	6.0	0.0	3.5	0.7

E. vermicularis, however, was recovered from 156 or 8.1 per cent of the school children and from 9, 2.0 per cent, of the positive family members. Infections by *Strongyloides stercoralis* and *Trichuris trichiura* were both found in less than one-half of one per cent of those examined. Two infections by *Hymenolepis nana* were found, both in the same family.

The observed prevalence of *Enterobius vermicularis* was unexpectedly high since the methods of examination used in the survey were not suitable for the demonstration of this parasite and diagnosis depended upon recovery of adult worms or eggs from the fecal specimens. Consequently, the observed prevalence cannot be accepted as even approaching the true prevalence.

Analysis of the distribution of the various parasites by race and residence, whether urban or rural, reveals some interesting differences in the prevalences of infection observed among the schoolchild and positive family groups. The data are shown in Table 4. Since the negative control group was urban, it is omitted from this table.

Prevalences of pathogenic protozoa among the school children show no significant differences between races or place of residence. The most striking observation is the almost identical prevalence of *E. histolytica* in three of the four groups of children: 6.0 per cent among the white urban; 6.9 per cent among the Negro urban; and 6.3 per cent among the rural Negro children.

The highest prevalence of *E. histolytica* among the positive families was found among the rural whites, 27.0 per cent. The lowest prevalence, 17.2 per cent, was observed among the urban white families. Although the urban-rural difference approaches significance for whites, when total urban families are compared with

FIG. 1. No. 10: Rural Negro (K). 10 of 12 infected by E.h. No privy. Water supply surface seepage below defecation area.

FIG. 2. No. 20: Urban Negro (W). 5 of 6 infected by E.h. Well and privy closely approximated. Water known to be contaminated. Grade 4 sanitation.

total rural families, the difference is not significant. Although infections by this ameba were somewhat more frequent among the Negroes, the difference between races is not statistically significant.

Infections by *Dientamoeba fragilis* among positive family members were somewhat more frequent among urban residents than among rural, but not significantly so. Infections by *Giardia lamblia* were more frequent among urban residents regardless of race but the differences are not statistically significant.

Analysis by race and residence of infections by the two most frequently observed commensal protozoa showed some statistically significant differences in in distribution. *E. coli* was more common among the colored children than among the white. In the former it was present in 29.0 per cent and in the latter group in 20.2 per cent. This difference is highly significant. Within the individual racial groups, moreover, the distribution was relatively uniform. Although the prevalence of this commensal was somewhat higher in the rural population than among the city children, it is not significantly so.

In the positive family group, *E. coli* was found most frequently among the rural families. The prevalence was identical, 38.8 per cent, in both the colored and the whites. The prevalence of 22.6 per cent among the urban Negro families is not significantly higher than that of the white urban families, 19.8 per cent. Both these values are below the prevalence of the respective rural rates of infection, pointing out thereby the rural nature of this infection.

Endolimax nana was quite uniformly distributed among the children irrespective of race or family environment. Among the positive families, on the other hand, infections by *Endolimax nana* were more frequent among urban families,

FIG. 3. No. 25: Rural White (M). 6 of 11 infected by E.h. Open well down hill from privy. Grade 4 sanitation.

FIG. 4. No. 41: Urban Negro (D). 4 of 6 infected by E.h. Grade 1 sanitation.

in which 24.0 per cent were found infected. In contrast, 26.0 per cent of the rural families were infected. This difference between urban and rural families approached significance to the extent that it suggests a real difference. No significant racial difference was noted.

FIG. 5. No. 51: Urban White (P). 4 of 4 infected by E.h. No toilet facility. Grade 4 sanitation.

All infections by *Iodamoeba bütschlii* were found among rural residents. The prevalences of the other commensal protozoa was relatively uniform in distribution and too few in number to show significant differences.

With the exception of *Enterobius vermicularis*, all helminthic prevalences were low. The majority of the hookworm infections were found in rural residents, mostly among white individuals. The highest prevalences of *Ascaris lumbricoides* infections were also found among rural residents in both groups. There was little difference between races among the school children, whereas 9 of the 10 infections among positive family members were found in the white families. Both *Strongyloides stercoralis* and *Trichuris trichiura* were found only among the school children and were too few in number to show statistically significant differences.

Analysis of the data relative to *Enterobius vermicularis* infections revealed a marked racial difference in the distribution of this helminth; 13.4 per cent of the infections were among the whites and 2.7 per cent among the Negroes. This racial distribution pattern conforms to the observations of others (Einhorn & Miller, 1946 and Cram, 1943). The inadequacy of the data appear, however, when the urban-rural distribution is examined. Contrary to the accepted pattern, the observed infections by this parasite were nearly twice as frequent among the rural children as among the urban. Although infections were less frequently found among the positive family members, this same observation holds true.

DISCUSSION

This survey of the prevalences of intestinal parasitic infections in Forsyth County, North Carolina was based upon a sample of 2,063 children in Grades

II, III, IV and V in the public schools selected to give competent distribution by race and residence. Of these, a total of 1,934 were examined. An additional group of 609 persons, comprising family members of the school children, was also examined. The cooperation rate for the school children was 93.7 per cent and, for the total group of children and family members, it was 91.7 per cent.

Infections by one or more of the intestinal parasites were demonstrated in 52.2 per cent of the children and in 60.1 per cent of the family members of children found to be infected by *E. histolytica*. Contrasting with these figures, the prevalence among family members of the children not found infected by *E. histolytica* was 24.4 per cent. This difference is highly significant. Without exception, infections by the intestinal protozoa were more numerous among the positive family members than among the entire group of school children or among the members of the negative control families.

The observed prevalence of *E. histolytica* among the school children was 6.1 per cent. Owing to the fact that a simple random sample of the entire population of Forsyth County was not feasible, precise estimation of the overall prevalence of this parasite is not possible. An approximation has been attempted by the following method.

School children and their family members were used to constitute the population under investigation ("target population") from which the sample was drawn. Persons who had no children in school or who were not living in households where there was a schoolchild, were automatically excluded from this "target population." The sampling plan was not representative in this respect. Based upon studies of family size, between 15 and 20 per cent of the county population was thus excluded. If this group of households without school children did not differ in rate of infection from the other group, no bias would be introduced in estimating prevalence. Such was probably true but impossible to prove from the data available and the estimates of the prevalence rate should be limited to the 80 to 85 per cent of the total County population with a child in the public schools.

The procedure is to predict that percentage of the "target population" which would have had positive findings from a single stool examination. This percentage is then adjusted by dividing the efficiency factor (0.59) into it to give an estimate of the true prevalence rate.

The percentage of the "target population" which would have been positive for *E. histolytica* at a single stool examination is found by a weighting scheme as follows:

$$\left\{\begin{array}{l}\text{Percentage of school children who were positive}\end{array}\right\} \times \left\{\begin{array}{l}\text{Percentage of positive family members of positive school children}\end{array}\right\} - \left\{\begin{array}{l}\text{Percentage of school children who were negative}\end{array}\right\} \times \left\{\begin{array}{l}\text{Percentage of positive family members of negative school children}\end{array}\right\}$$

This procedure cannot be used for the rural population since it was not practicable to examine family members of negative rural school children. Thus, the last or fourth item of the above formulation is unknown and a valid estimate of the true prevalence cannot be made from the data pertaining to the rural areas.

The calculated true prevalence of *E. histolytica* in the urban section of Forsyth County was 3.3 ± 1.2 per 100 persons. Although the rate for the Negro urban population was two and one-half times as great as that for the white population, the difference between the races is not statistically significant and may be a chance observation.

The available data permit only an informed guess concerning the true prevalence in the rural sections. This is a rate of 4.2 per 100 persons. The standard error of estimate would probably be of the same order of magnitude as that for the urban areas. The difference between urban and rural is not significant. The individual racial prevalences are estimated to be 3.4 per 100 persons for the rural whites and 5.1 per 100 persons for the rural Negroes. Here again, the difference is not statistically significant.

Except for *Enterobius vermicularis*, helminthic infections were infrequently found. The observed overall prevalence of 0.8 per cent for hookworm infection and of 1.1 per cent for infection by *Ascaris lumbricoides* are maximal prevalences because a large segment of the total persons observed were selected positive family members. Thus, the marked drop in prevalence of these infections reported by Keller *et al.* (1937) and by Otto and Cort (1934) have continued and they no longer constitute a public health problem in Forsyth County. This change in conditions is unquestionably the result of the enforcement of the sanitary privy law and the almost complete elimination of fecal soil pollution.

The prevalences of the intestinal protozoa observed in the course of the survey, however, demonstrate conclusively that infections of fecal origin are still widespread. It is apparent that the standard type privy, widely recommended and used in rural areas, has resolved only a part of the problem presented by intestinal parasitic infections and that the further development and improvement of this type of sanitary installation is necessary before fecal contamination is eliminated.

CONCLUSIONS

1. Intestinal parasitic infections were found in 52.2 per cent of 1,934 school children who constituted a representative sample of the total school population in Grades II through V in Forsyth County, North Carolina.

2. The observed prevalence of infection by *Entamoeba histolytica* in the schoolchild sample population was 6.1 per cent.

3. The calculated true prevalence of *E. histolytica* in the urban section of Forsyth County was 3.3 ± 1.2 per 100 persons.

4. Examination of 449 family members of the children found infected by *E. histolytica* revealed a prevalence of 60.1 per cent of intestinal parasitic infections in contrast to a prevalence of 24.4 per cent among the 160 members of the negative control families.

5. The virtual disappearance of hookworm infection and of *Ascaris lumbricoides*, together with the high prevalence of infection by the intestinal protozoa, indicate that soil pollution has been largely eliminated but that the standard type privy has not controlled the spread of infections by the protozoa.

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THE EFFECT OF RABBIT ANTISERA ON THE ABILITY OF *ENTAMOEBA HISTOLYTICA* TO PHAGOCYTOSE RED BLOOD CELLS[1]

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Little is known of the fundamental nature of *Entamoeba histolytica*. Its critical growth requirements and characteristics, although under intensive study, have not yet been elucidated. The nature of the media now available for propagation of these amebas has made it difficult, and in some cases impossible, to study such critical things as strain variations, pathogenesis, antigenic structure, etc. Variations of certain types have been described among different strains of *E. histolytica* (Faust *et al.*, 1946; Shaffer, 1952; Meleney and Frye, 1935; Frye and Meleney, 1938). The best recognized variation involves the size of the cysts and trophozoites, and this has led to a division of the species into large and small races. The true significance of this division is not yet known, although some workers feel that the small race strains are nonpathogenic. Others have presented evidence which does not completely support this belief (Frye and Meleney, 1938). Other strain variations involve differences in growth curves and in pathogenicity for laboratory animals. It is not certain, however, whether or not the observed differences in pathogenicity are inherent characteristics of the amebas or are the result of the influence of an associated bacterial flora. In connection with attempts to develop satisfactory serological aids for the diagnosis of amebiasis, it has been postulated that one of the greatest deterrents might be antigenic variation between strains of *E. histolytica*. No specific information has been available on this point and the postulations are based on the failure of antigens made from one or two strains of *E. histolytica* to give a greater per cent of positive tests with the serum of patients known to have amebiasis.

Any specific information indicating antigenic variation among strains of *E. histolytica* would be of great value to those interested in diagnostic aids in amebiasis as well as of great interest in the epidemiology and immunology of the disease.

The demonstration that the serum of rabbits which had been inoculated with suspensions of a specific strain of *E. histolytica* was capable of markedly inhibiting the ability of this and other strains of the amebas to phagocytose rabbit RBC (Shaffer and Balsam, 1954) seemed to offer an opportunity to study possible variations between different strains of the organism in a manner not previously available. It was thought possible that this might offer a means of detecting antigenic differences.

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In a preliminary report (Shaffer and Balsam, 1954), it was shown that the ability of *E. histolytica* to ingest rabbit RBC was a constant characteristic and that the phagocytic index could be standardized. It was further found that, under specified conditions, certain agents, such as drugs and specific antiamebic sera, exerted a definite and measurable effect on this ability. No further experiments have been done using drugs, but an extensive study of the effects of strain-specific antisera is in progress. This report deals with the initial phase of this investigation, involving the production of strain-specific rabbit sera and the effect of these sera on the homologous and on heterologous strains of *E. histolytica*.

MATERIALS AND METHODS

In all, seven strains of *E. histolytica* have been employed. They have been maintained exclusively in the S-F medium which has been described previously (Shaffer *et al.*, 1948, a & b). The various strains of *E. histolytica* came originally from widely scattered sources as follows: strains 200 and 103 were originally obtained by us from Mr. M. C. McCowen of the Lilly Research Laboratories who had, in turn, received them from Dr. C. W. Rees of the National Institutes of Health; strain LUNA was isolated, using S-F medium, from the stool of a patient with amebic dysentery, in Nashville, Tennessee in 1948; strains K-9 and K-14 were obtained from Dr. M. M. Brooke of the C.D.C. and were brought from Korea by Dr. W. W. Frye in 1951; strain COOK was isolated in 1953, using the S-F medium, from the stool of a patient with amebiasis at the Cook County Hospital, Chicago, Illinois; strain SHELTON was isolated in 1954 by inoculation of S-F medium with a portion of human liver abscess obtained at autopsy. The size of all of these strains is within the range accepted as large race. No clone isolations were made with any of the strains used for the experiments to be described. It was thought that this was more desirable under the circumstances, although clone isolation may become desirable as the work extends.

Five of the strains of *E. histolytica* were inoculated into rabbits to produce antisera. So far, no sera are available for SHELTON and K-14. The method for production of the sera for testing was as follows: Two young, healthy, adult rabbits of either sex and weighing 5–7 lbs., purchased from a local breeder, were used with each strain of *E. histolytica*. Preliminary bleedings by cardiac puncture were made and the sera stored in the deep freeze at −15°C. The rabbits were then given, at 3 to 4 day intervals, a series of 8 intravenous inoculations with suspensions of the appropriate strains of amebas. These suspensions were made in the manner described previously (Shaffer and Balsam, 1954). Each dose consisted of 1.0 ml. containing 600,000 to 3,000,000 organisms; thus, each rabbit received several million cells during the course of the inoculations. Seven to 10 days after the final inoculation, blood was collected and the serum stored as before. After a rest of 2 to 3 weeks, the rabbits were given a single "booster" inoculation similar to those of the original series and bled after 5 to 10 days. Thus, for each rabbit, there were 3 serum samples available for testing.

Two control groups of 2 rabbits each were included. One group was given a series of 8 intravenous inoculations with the sediment from the S-F medium and

the other group was given a similar series of inoculations, each of which was 1.0 ml. containing upwards of 2 billion cells of the gram negative anaerobic streptobacillus used in the preparation of the S-F medium. These animals were bled and given "booster" inoculations in the same manner as were those receiving the *E. histolytica*.

Preliminary experiments were done on the normal and anti-amebic sera of selected rabbits to determine whether or not complement was necessary for inhibition of the phagocytosis. Portions of the rabbit sera were heated in a 56°C. water bath for 30 minutes and tests run in parallel with unheated portions of the sera. No differences in activity were seen. Furthermore, the addition of fresh guinea pig serum, as much as 0.1 ml. of undiluted serum to 2.0 ml. of antiserum-ameba mixture, had no enhancing effect on the phagocytosis-inhibiting activity of the heated serum. Subsequently all sera were heated at 56°C. for 30 minutes before use.

Further experiments showed that the pH of the medium exerted considerable influence on the phagocytic index of the amebas. In a typical experiment with cultures of strain K-9 adjusted to varying pH, it was found that the phagocytic index at pH 5.4 was 1.9; at pH 6.0 it was 7.2; at pH 6.5 it was 8.8; at pH 6.9 it was 10.5; and at pH 7.6 it was 2.4. Other strains of *E. histolytica* had similar ranges under these circumstances. All pH adjustments were made with HCl or NaOH and checked with the Model H-2 Beckman, line operated pH meter. Thus, it will be seen that good phagocytosis was obtained between pH 6.0 and 6.9, but above or below this range, the amebas rapidly lost their ability to ingest RBC. It was necessary in all experiments to check the pH of the cultures to make sure all were the same in a given experiment and fell within the above range. Fortunately, it was seldom necessary to make adjustments, since all cultures in a given series tended to have the same pH and, when satisfactory growth occurred, the pH was almost invariably 6.2 to 6.3. The addition of the normal or anti-amebic rabbit serum to accomplish the 1:8 dilution described below raised the pH about 0.25 pH. Thus, the experiments to be described were done at pH 6.45 to 6.55.

The serum specimens from each rabbit were tested individually against all seven strains of *E. histolytica*. The following technique was used throughout: 48 hour cultures of the appropriate strains of *E. histolytica* showing 4+ amebas (Shaffer and Balsam, 1954) were utilized in all cases. Previous attempts at exact standardization of the number of amebas showed that the manipulations necessary to accomplish this damaged the cells to the extent that phagocytosis was irregular. Thus, the only standardization in these experiments was accomplished by choosing tubes containing approximately similar numbers of trophozoites as judged by direct microscopic examination of the cultures. The petrolatum seal was broken and culture fluid carefully removed, leaving 1.75 ml. in the tube. To this was then added 0.25 ml. of the serum to be tested for phagocytosis-inhibiting ability. Thus, the serum was diluted 1:8 in all cases. It was indicated previously (Shaffer and Balsam, 1954), and was later confirmed, that this was the most desirable serum dilution to use. Since the addition of serum tended to alter the

pH, it was thought best to use a constant serum dilution. Otherwise it would be necessary to adjust the pH with more HCl or NaOH and it was desirable, as indicated previously, to avoid undue manipulation of the cultures. It may be necessary to change the dilution if higher titered sera can be obtained. After addition of the serum, the mixture was agitated gently, re-sealed with petrolatum and placed in the 37°C. incubator for 2 hrs. Two hours was chosen as a standard incubation time in these experiments after preliminary tests indicated this to yield consistent results. The tests were done using 30, 60, 90, 120, 180 and 240 minute exposures to immune serum, with controls in which Hank's balanced salt solution was used in place of the serum. The results with 60, 90, 120 and 180 minutes incubation were similar. Thirty minutes gave irregular results in all tubes, apparently because the amebas did not have sufficient time to recover from the damage resulting from mixing after the addition of Hank's solution or serum. The amebas tended to deteriorate somewhat in the 240 minute incubation period. At the end of the 2 hr. period, the petrolatum seal was again broken and 0.3 ml. of a 1:10 dilution of defibrinated rabbit blood was added with gentle, but complete, mixing. The tube was again sealed and replaced in the 37°C. incubator for 15 minutes. On removal from the incubator this time, the seal was opened as widely as possible by pressing the petrolatum against the sides of the tube. Ten ml. of distilled water was then added gently in order to hemolyze all unphagocyted RBC. By means of a capillary pipette, samples were removed from the tube, placed on a clean slide, and a cover slip applied. The number of RBC in each of the first 25 amebas seen was recorded and the average calculated as the phagocytic index. Each determination consisted of seven tubes, two with the normal sera of the two rabbits, two with the anti-amebic serum, two with "booster" serum, and one control in which Hank's solution was substituted for the serum. It was desirable that all sera of the two rabbits of a pair were tested on the same day with similar cultures.

All dilutions in these experiments were made with Hank's solution and all reagents were brought to 37°C. before use. The rabbit blood was collected by cardiac puncture and defibrinated by shaking with glass beads. It was never used if more than 6 days old.

These experiments, in addition to providing data on the phagocytosis-inhibiting ability of the various sera, have also provided certain other information of interest. Thus, it has been possible to study and tabulate the distribution of RBC in the individual amebas over a series of experiments and to study variations in the ability of the different strains of *E. histolytica* to ingest RBC.

RESULTS

Table 1 shows the average phagocytic index of each of the 7 strains of *E. histolytica* used in the experiments. It also shows the limits of variation observed in day to day experiments with a given strain. The averages were calculated from 10 or more determinations with each strain, each done on a different day. The range of variation represents day to day differences, not tube to tube variation in any single run. Actually, on similar cultures of a single strain of *E. histo-*

TABLE 1

Average phagocytic indices of 7 strains of Entamoeba histolytica *when exposed to rabbit rbc for 15 min. at 37°C.*

Strain	Average index*	Range of variation
200	12.7	11.6 to 13.8
Luna	12.5	10.6 to 14.0
103	10.5	9.6 to 11.9
K-9	8.3	6.6 to 10.8
K-14	7.6	6.4 to 9.3
Cook	7.1	5.3 to 9.2
Shelton	4.5	2.6 to 6.0

* All determinations used to calculate the averages were done without the addition of serum to the cultures.

lytica done on a single day, the total variation in index has never been more than 10 per cent. The day to day variation may be greater than this if the pH is not controlled. In all these experiments, the pH fell between 6.4 and 6.7.

It will be seen that each of the 7 strains of *E. histolytica* has a characteristic range of phagocytic activity under the conditions used, ranging from the SHELTON strain with 4.5 to the 200 strain with 12.7. These have remained constant in the course of this work.

Table 2 shows the results of the phagocytosis-inhibition tests with the sera of rabbits inoculated with 5 of the strains of *E. histolytica* titrated against all 7 strains. Each of the figures shown in the "N" (Normal Serum) column is the average for the two rabbits used for inoculation with the particular strain of amebas and each figure in the "I" (Immune) column is the average of 4 sera, two from each rabbit. As indicated before, one sample of serum was collected from each rabbit after the course of 8 inoculations and the second was collected after the "booster" inoculation. It was found that there was no significant difference between the two sera from any rabbit. As shown in the table, all determinations were done with the serum diluted 1:8.

An examination of the table reveals that in all cases the homologous anti-amebic serum markedly reduced the phagocytic index as compared with the normal serum, the per cent reduction varying from 72 with strain 200, to 85 with strain 103. It was felt that the per cent reduction using standard amounts of serum was better than attempting to establish serum titers, since this might, as indicated before, introduce pH variations that would be difficult to control. Further inspection reveals that some heterologous systems also resulted in marked reduction of the phagocytic index, thus the anti-200 serum reduced the index of strain 103 by 46 per cent, strain LUNA by 68 per cent, and strain SHELTON by 63 per cent, but the anti-200 serum had doubtful activity against strain COOK, little or none against K-9 and K-14. The anti-sera for the other three strains showed less heterologous activity. Strain K-9 is of particular interest since this strain was little affected by the sera produced with the other four strains and its anti-serum did not markedly affect any of the other 6 strains. Strains

TABLE 2

Phagocytic indices obtained with the normal and "immune" serum of rabbits inoculated with various strains of Entamoeba histolytica

Serum tested against strains	Phagocytic indices of serum of rabbits inoculated with *E. histolytica* strains																			
	200				103				Luna				Cook				K-9			
	C*	N†	I‡	% reduction§	C	N	I	% reduction	C	N	I	% reduction	C	N	I	% reduction	C	N	I	% reduction
200	12.2	8.6	2.4	72	13.7	14.1	5.4	62	13.8	12.6	5.9	53	13.1	12.3	7.6	38	13.2	13.3	10.7	20
103	9.7	9.7	5.2	46	11.9	11.8	1.9	85	9.8	9.1	5.1	44	10.2	10.0	4.1	59	9.6	8.4	5.6	33
Luna	11.9	11.2	3.6	68	10.6	10.3	3.1	70	14.0	12.8	2.1	84	13.2	12.1	9.9	18	12.8	11.2	7.0	38
Cook	5.3	4.8	3.2	33	6.9	5.4	1.2	78	8.4	6.8	5.1	25	6.8	7.1	1.5	79	7.4	6.4	4.8	25
K-9	6.7	7.0	6.3	10	8.4	7.9	7.2	9	8.2	7.6	5.4	29	8.0	9.0	6.0	33	6.6	7.1	1.3	82
K-14	9.3	7.7	5.8	25	7.8	8.8	6.2	30	9.1	7.2	5.3	26	6.7	5.1	2.1	60	9.1	7.7	4.8	38
Shelton	5.5	5.6	2.1	63	3.1	3.0	1.2	60	4.6	4.6	4.1	11	3.1	3.2	0.7	78	6.0	5.3	4.5	15

* Control with Hank's BSS substituted for serum.

† Normal serum—final dilution 1:8.

‡ "Immune" serum—final dilution 1:8.

§ Percent reduction in phagocytic index between normal and "immune" sera.

TABLE 3

Phagocytic indices obtained with normal and "immune" serum of rabbits inoculated with the sediment of S-F medium and with the streptobacillus

Strain of *E. histolytica*	Phagocytic indices of serum of rabbits inoculated with							
	S-F medium				Streptobacillus			
	C	N	I	Per cent reduction	C	N	I	Per cent reduction
200	13.0	12.7	12.6	0.79	13.1	10.8	10.1	6.5
103	11.2	9.9	8.1	18.2	11.0	6.1	5.8	4.9
Luna	12.6	9.9	9.0	9.1	12.4	11.3	8.5	24.8
Cook	7.9	7.4	6.0	18.9	9.2	6.5	5.1	21.5
K-9	10.3	10.1	9.0	10.9	10.8	9.3	9.1	2.1
K-14	8.3	7.0	5.5	21.4	6.4	7.4	6.1	17.6
Shelton	4.6	3.8	4.0	+5.3	2.6	2.7	2.4	11.1

Legend same as Table 2.

200 and 103 show the greatest heterogeneity, with LUNA and COOK following in that order, and K-9 standing by itself. No definite statement can be made about K-14 and SHELTON in the absence of specific anti-sera, but it is likely that K-14 may be quite different.

Table 3 records the results obtained using the serum of rabbits inoculated with the sediment from S-F medium and with the gram negative anaerobic streptobacillus used to prepare the S-F medium. It will be seen that some reduction in phagocytic index occurred in most cases, varying from 0.79 to 25 per cent. In one case, strain SHELTON, there was a higher index with anti-serum than with normal serum.

TABLE 4

Relationship among various strains of Entamoeba histolytica *as indicated by phagocytosis-inhibition*

Strain	Antisera against strains				
	200	103	Luna	Cook	K-9
200	+*	+	+	±	0
103	+	+	+	+	±
Luna	+	+	+	0	±
Cook	±	+	0	+	0
K-9	0	0	0	±	+
K-14	0	±	0	+	±
Shelton	+	+	0	+	0

* + = positive, 40 per cent or more reduction in phagocytic index; ± = doubtful, 30 to 40 per cent reduction in phagocytic index; 0 = negative, less than 30 per cent reduction in phagocytic index.

Table 4 represents an attempt to tabulate the relationships revealed by Table 2, while taking into consideration the results shown in Table 3. In this table, a positive (+) was recorded if the anti-serum reduced the phagocytic index of a particular strain by 40 per cent or more over the normal serum, a doubtful (±) if the reduction was between 30 and 40 per cent, and negative (0) if the reduction was less than 30 per cent. On this basis, it will be seen that (1) cross relationships exist between strains 200, 103 and LUNA, (2) K-9 appears to be in a class by itself, (3) COOK shows cross relationship with 103 and possibly K-14 and SHELTON, and (4) SHELTON is possibly related to 200, 103 and COOK.

DISCUSSION

The present studies have offered an opportunity to study the distribution of phagocytic activity amongst the cells of the various *E. histolytica* strains. Great variation occurs in the ability of the amebas to ingest RBC. With strain 200, which has an average index of 12.7, the number of RBC per ameba varies from 0 to as many as 35. Despite this wide variation, the average number of RBC per ameba has proven to be quite consistent as indicated in Table 1. Plotting the distribution of RBC in the amebas has invariably resulted in a normal curve, with the peak occurring at the point corresponding to the phagocytic index. With strains of lower phagocytic index, the spread becomes progressively less, but the shape of the distribution curve remains essentially the same. It is thought possible that the phagocytic activity of the individual ameba may reflect its stage of development, thus the curve just mentioned may represent developmental distribution within the culture. A further possibility is that there are inherent or genetic differences in the amebas within the culture, some capable of ingesting large numbers of RBC and others capable of ingesting only a few RBC, thus one would be measuring a population distribution. In either case, it is not surprising that the phagocytic index, calculated from the observation of 25 randomly selected amebas, should prove to be quite constant if the population

of the amebas were produced under controlled conditions. Further studies of the fundamental nature of the phenomenon are in progress and a more complete description will be published at a later date.

The nature of the activity of the rabbit anti-serum on the individual *E. histolytica* trophozoites is not yet known. In a previous paper (Shaffer and Balsam, 1954), it was suggested that antibodies directed against surface antigens might be responsible for loss of motility which would affect the ability of the amebas to ingest particles. Cole and Kent (1953) have described an immobilization test with anti-amebic serum. It seems likely that antibodies are involved, but their activity may not be limited to the surface of the amebas. It may be that some internal structure or structures are affected.

The results shown in Table 2 have revealed considerable evidence of variation among the 7 strains of *E. histolytica* studied so far. As mentioned previously, it seems likely that antobides are involved in these observations and, if so, there is indicated considerable antigenic variation between some of the strains. The K-9 strain appears to be in a class by itself as measured by this technique, and K-14 shows evidence of considerable variation from the other strains. In no case did any strain appear identical with any other strain. More observations need to be made on the inherent technical variation in the methods being employed before exact evaluations of strain differences can be attempted.

The results obtained with the serum of rabbits inoculated with the gram negative anaerobic streptobacillus and with the uninoculated S-F medium, as shown in Table 3, are interesting from at least two standpoints. First of all, the differences observed between normal and anti-amebic serum from strain to strain may indicate the range of variation in the technical procedures. Other observations indicate that this is probably not the explanation, but further experiments are necessary on this point. A second possibility is that there may be some antigenic relationship between the streptobacillus or some other medium constituent and the amebas, greater in some cases than others, thus accounting for the differences in results with the different strains. Pending an answer to this problem, reductions in phagocytic index of up to 25 to 30 per cent cannot be accepted as significant when testing the sera.

Preliminary experiments indicate that the phagocytosis-inhibiting activity of the rabbit serum can be completely removed by absorption with a concentrated suspension of washed, homologous *E. histolytica* trophozoites. This is interpreted as further evidence that antibodies are involved. It now is hoped that by means of appropriate cross-absorption tests it will be possible to tabulate the antigenic relationship between the various strains of *E. histolytica* now on hand and extend these studies to strains obtained from other sources. These experiments may provide answers to some of the problems raised above regarding technical variations in the methods.

SUMMARY

The effect of strain-specific rabbit anti-serum on the ability of cultures of *E. histolytica* to ingest rabbit RBC has been studied. These sera have shown

marked ability to inhibit the phagocytic activity of the homologous strain of *E. histolytica* and varying ability to inhibit the phagocytic activity of heterologous strains. One strain, K-9, appears to be quite different from six other strains when tested in this way. The other six strains have exhibited varying ranges of heterogeneity. It is suggested that these results indicate antigenic variation among strains of *E. histolytica*. Other experiments are necessary to provide final proof.

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GLUCOSAMINE IN THE CULTURE OF *ENTAMOEBA HISTOLYTICA* WITH A MIXED BACTERIAL FLORA

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It was shown in a previous publication (Greenberg, Taylor, and Bond, 1954, 1955) that *Entamoeba histolytica* would grow in a liquid medium containing salts, trace minerals, egg albumin, rice powder, and any one of a number of organic amines or amino alcohols. If the amines were absent, or if non-nitrogenous organic compounds or even some amino acids were substituted for them, the cultures failed to survive more than a few transfers.

We have now examined the specificity of the requirement for commercial egg albumin. Certain of the results obtained in these studies have necessitated a re-examination of the non-protein nitrogen sources required for the growth of the amebae.

METHODS

While the methods used in the present studies were similar to those used previously (Greenberg *et al.*, 1954, 1955), some changes were made. The basal salt medium used and the methods of inoculation, transfer, and assay for viability were the same. The time of autoclaving at 15 pounds pressure was reduced to 8 to 10 minutes.

A medium was considered "successful" if it supported the growth of amebae through 15 transfers made every other day. Media prepared at the beginning of an experiment were kept after sterilization, at room temperature, with no special precautions against exposure to light. After inoculation, the cultures were maintained in an incubator at 37° C. Cultures were examined for the presence of viable amebae at each transfer. They were discarded when no amebae were observed for two successive transfers.

RESULTS

Two lines of investigation were being conducted during the same period of time. Results obtained in one line of investigation were transferred to the other, and the reverse. The data have been arranged for convenience in two categories: (1) the effect of changes in the proteins or protein products on survival of amebae (Table 1), and (2) the effect of changes in the non-protein nitrogen products in media containing gelatin as the protein (Table 2).

It was found (Table 1) that in the presence of glucosamine hydrochloride (2.5 g./liter), bovine serum proteins would substitute for egg albumin in the culture of amebae, but casein, blood fibrin, lactalbumin, and edestin would not. The proteins which did not support the growth of the amebae through 15 transfers were not completely solubilized, which may account for the failures. All the above proteins were tested at 2.5 grams per liter and at pH 7.2 to conform to our

TABLE 1

The cultivation of Entamoeba histolytica *in a basal medium plus glucosamine HCl (2.5 g./liter) with various proteins and protein products*

Substrate	Concentration g./liter	No. trials	No. transfers with living amebae
Gelatin	2.5	1	15
Gelatin	1.25	3	15, 15, 15
Casein	2.5	2	5, 6
Edestin	2.5	1	5
Lactoalbumin	2.5	1	8
Blood fibrin	2.5	1	5
Bovine plasma albumin	2.5	1	15
Bovine plasma Fraction III-I	2.5	1	15
Thiotone	1.25	1	15
Myosate	1.25	1	15
Lactalysate	1.25	1	7
Trypticase	1.25	2	15, 15
Phytone	1.25	1	15
None	—	4	5, 5, 3, 1
Glycine	2.5	2	6, 5
Amino acids*	—	1	4

Thiotone, myosate, lactalysate, phytone, and trypticase from Baltimore Biological Laboratories. Bovine plasma proteins from Armour & Co. Gelatin from Fisher Scientific Company. Casein, edestin, lactalbumin, blood fibrin, glycine from Nutritional Biochemicals.

* In mg./liter. Arginine 110, histidine 10, lysine 65, phenylalanine 49, methionine 16, threonine 66, leucine 45, isoleucine 15, valine 34, glutamic acid 144, aspartic acid 120, glycine 336, α-alanine 129, proline 402. All amino acids were natural configuration; all were obtained from Nutritional Biochemicals Co.

previous experiments with egg albumin (Greenberg *et al.*, 1954, 1955). Under the same conditions, in fact in the same experiments, gelatin, solubilized by warming, substituted adequately for egg albumin. It was subsequently found that concentrations of gelatin of 1.25 and even 0.625 g./liter were adequate and in the remaining series proteins or their products were tested at 1.25 g./liter. It was also found that media containing gelatin gave more consistent results at pH 7.8 than at lower pH values, though the pH was not critical within a reasonable range (pH 7.7 to 7.9).

Because gelatin is considered a nutritionally incomplete protein, the success of the cultures containing this protein was of some interest to us. A medium containing gelatin and ammonium chloride, for example, would be the simplest medium so far devised for the cultivation of *E. histolytica*. Ammonium chloride (2.5 g./liter) did not support the growth of amebae beyond 3 transfers in the presence of gelatin (Table 2). A series of experiments was undertaken in which organic amines and amino alcohols were substituted for glucosamine in the presence of gelatin. In these tests, 4 to 6 media tubes, each containing a different substance (in one instance nothing), were inoculated at one time usually along with one tube containing glucosamine. Twenty-four substances were tested in

TABLE 2

Cultivation of E. histolytica *in a basal medium plus gelatin (1.25 g./liter) with various additional substrates*

Substrate (250 mg./100 ml.)	No. trials	No. transfers with living amebae
Ammonium chloride	2	3, 3
Nothing	1	0
Diethylamine	2	1, 6
Triethylamine	2	0, 0
Di-n-propylamine	1	1
Ethanolamine	4	0, 9, 1, 2
Triethanolamine	3	3, 0, 2
Dimethylethanolamine	1	0
1-Amino-2-propanol	3	9, 7, 1
2-Amino-2-methyl-3-propanediol*	3	2, 0, 2
2-Amino-2-ethyl-1,3-propanediol*	1	3
2-Amino-2-methyl-1-propanol*	2	0, 1
1-Amino-2-butanol	1	1
2-Amino-1-butanol	3	0, 1, 0
4-Amino-2-butanol	2	6, 2
1-Amino-2-pentanol	3	2, 0, 0
Tris(hydroxymethyl)aminomethane*	3	6, 5, 3
Choline chloride	2	0, 2
D(+)-Glucose	1	1
D(+)-Glucosamine hydrochloride†	5	15, 15, 11, 15, 15
N-Methylglucamine*	2	0, 4
L-Asparagine	1	1
L-Malic acid	1	3
Pyridine	2	0, 1
β-Picoline	2	0, 0

* Kindly furnished by Commercial Solvents Corporation.

† Results do not include those from Table 1.

52 media, and 4 of these, all containing glucosamine, underwent 15 successful transfers. In one test with glucosamine, amebae disappeared after the eleventh transfer. In 2 of the remaining 46 tests the amebae survived 9 transfers (1-amino-2-propanol and ethanolamine) and in a total of 6 they survived more than 5 transfers. In over half of the tests (27), the amebae failed to survive more than one transfer. About 90 per cent of the cultures, exclusive of those containing glucosamine, failed to survive more than 5 transfers, and none survived over 9 transfers.

Additional tests on the specificity of the protein requirement were conducted in the presence of glucosamine at 2.5 g./liter. Media with glucosamine alone or in the presence of glycine or of a mixture of amino acids simulating gelatin (7 tests, Table 1) failed to maintain the amebae beyond 6 transfers. Five commercially available digests of proteins were tested; all but lactalysate were able to maintain the amebae through 15 transfers.

DISCUSSION

The system with which we have been working is obviously complex. It cannot be assumed without additional evidence that the observations made in the present

experiments would have general application. The simplified media which have been devised may be successful for the cultivation of a particular strain of amebae (200) with a particular group of bacterial associates. It might, however, be profitable to attempt the simplification of the basal medium used for the cultivation of *E. histolytica* with single, relatively non-demanding bacteria such as *Escherichia coli* (Dobell, 1947; Rees *et al.*, 1953), *Bacillus subtilis* (Chinn *et al.*, 1942), or *Aerobacter aerogenes* (Rees *et al.*, 1953). Hitherto, attempts to define the basal medium have been confined to cultures containing organism "t" (Hansen and Anderson, 1948; Hallman *et al.*, 1950), which itself requires many growth factors. This imposes an obvious restriction on the information which can be obtained about the amebae.

The simplest "successful" media we have devised to date contain inorganic salts, rice powder, a protein or protein derivative, and an additional organic substrate. The salt mixture may or may not be specific since the absolute requirements or the limits of tolerance have not been ascertained for any of them. The rice powder appears to be essential because in preliminary trials in which it was eliminated from otherwise adequate media, little or no growth of the amebae occurred (unpublished). Some form of protein or protein-like material was needed (in addition to the protein of rice powder) and an additional organic substance was required for growth of the amebae. There appeared to be an interrelationship between the type of protein used and the specificity of the organic substance which would satisfy the needs of the amebae for growth. When commercial egg albumin was used as a source of protein, any of a number of aliphatic amines and amino alcohols would serve as a second substrate; non-amino organic substances and probably amino acids could not (Greenberg *et al.*, 1955). When gelatin was used as the protein, only glucosamine hydrochloride, of the 21 nitrogen-containing substances tested, supported the growth of the amebae. Gelatin contains no tryptophane and is a poor source of other amino acids considered "essential." It would seem, therefore, that as the protein was made nutritionally inadequate, the requirement for the second substrate became more specific. On the other hand, gelatin contains no glucosamine, while egg proteins do. Under the conditions of the experiments, glucosamine would appear to be a requirement for the growth of the amebae. It is perhaps significant that most media devised for the cultivation of *E. histolytica* contain glucosamine as a part of a glycoprotein.

Substitution for gelatin of a single amino acid (glycine) or a mixture of amino acids produced unsuccessful media though several enzymatic digests of protein were adequate to support the growth of amebae in the presence of glucosamine. One can speculate that certain peptides are essential for growth or that trace impurities accompanying proteins are required. It should be noted that not all proteins were able to support the growth of the amebae (i.e., casein, lactalbumin, etc.).

As to the reason for the growth-promoting activity of glucosamine, one can offer only possible explanations: (1) it may act as a buffer as well as a source of energy and nitrogen for the amebae, the bacteria, or both; (2) it or its degradation products may be an essential for the growth of the amebae; (3) it may selectively promote the growth of beneficial bacteria or inhibit the growth of harmful

bacteria; (4) it may affect the metabolism of some or all of the bacteria in a manner conducive to the growth of the amebae. Whatever the explanation, further inquiries into the influence of this crystalline chemical substance on the growth of amebae would be profitable.

SUMMARY

Entamoeba histolytica was cultivated with a mixed bacterial flora through 15 transfers in 30 days in a medium containing inorganic salts, rice powder, glucosamine hydrochloride, and gelatin. The glucosamine could not be replaced by glucose, nor by any of a series of organic amines and amino alcohols. Gelatin could be replaced by serum proteins and the enzymatic digests of certain proteins. It could not be replaced by casein, edestin, lactalbumin, blood fibrin, nor by glycine or a mixture of amino acids based on the composition of gelatin.

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THE USE OF FUMAGILLIN IN THE TREATMENT OF AMEBIASIS

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Since the introduction of antibiotics, their use in intestinal amebiasis has been suggested by various workers in this field. Penicillin was used by Hargreaves (1945) in England and by Halawani *et al.* (1950) in Egypt, who found it effective in the treatment of several cases of severe amebic dysentery. Oxytetracycline (Terramycin) and Chlortetracycline (Aureomycin) were tried by Most and others (1950) in America and by Halawani (1950) and Nor El-Din (1951) in Egypt. The immediate results were very satisfactory, while the relapses after their use were fewer than after emetine and usually occurred at a later date. The value of the antibiotics in amebiasis is based on the assumption that they inhibit the associated bacterial flora which are supposed to be essential food for the amebae.

The value of the antibiotics depends also on the reduction of the tissue reaction to the bacterial infection, which permits the amebicides to reach effective concentration in the bowel. On the other hand other workers have suggested that chlortetracycline and oxytetracycline may have direct amebicidal action. Hewitt *et al.* (1950) reached this conclusion by excluding direct antibacterial action, when aureomycin was added in a high dilution to a culture of the ameba.

In 1949 a new antibiotic, fumagillin, was isolated from an aspergillus culture by Hanson and Eble (1949). In America, it was found that its activity on fungi, bacteria and viruses is very small, but it was found active against the ameba.

McCowen and his colleagues (1951) have established that it can inhibit a strain of *Entamoeba histolytica* growing in association with a mixed bacterial flora, in dilution as high as 1/130 million. In rats, cecal infection with *E. histolytica* was cleared by the administration of 12 mg. of the drug per kilo of body weight, given for two days and it was also found effective against amebiasis in rabbits. Hrenoff and Nakamura (1951) have found that fumagillin was very active in monkey amebiasis. The infected animal received 50–125 mg. of the drug per kilogram of body weight daily for 5 days and there was no recurrence on 8–14 weeks of observation. Parasitic relapse however occurred in 30 per cent of the monkeys in 1–2 months time. Results of the bromsulfalein test however showed liver damage when it was given in doses of 125 mg./kg.

Killough *et al.* (1952), working in the Naval Medical Research Unit No. 3 in Egypt, used the drug in 22 cases of amebiasis of which only one was an acute case. They used daily doses varying from 5–50 mg. given for a period of 14 days and reported disappearance of the parasites in all but the one acute case. However relapses occurred in most of the cases that were followed up. Anderson *et al.* (1952) had a similar experience with a group of chronic amebic cases.

THE PRESENT WORK

In the Research Institute, the trial of fumagillin was started in February, 1952. Being fully aware of the possible toxicity of the drug, routine kidney and

liver function tests were done carefully at the beginning and at the end of the course of treatment.

The hematologic data of the cases were thoroughly obtained, and any toxic symptoms such as dizziness or anorexia that were reported were very carefully observed. Various schemes of dosage were used. Routine daily stool examination for the ameba and sigmoidoscopy before and after treatment were done in all the cases. Progress sheets including the details of clinical data of the patients, the number of motions, their character, and the nature of the symptoms were carefully recorded.

The first group were given daily doses of fumagillin varying from 60–100 mg. for a period of 8–10 days. This comprised eleven patients suffering from acute dysentery at the time of admission and usually with a history of many recurrent attacks. Five of these cases did not show any clinical or parasitic remission. Their symptoms remained unabated while the vegetative amebae persisted in their stools for the whole period of 2 weeks' observation. I was compelled to use other drugs for the cure of the dysentery. These five cases included 3 patients who were given the smaller dose scheme of 60 mg. daily. Six of the cases improved clinically, while *E. histolytica* usually disappeared from the stool in an average time of 3–6 days. One of these relapsed within a month; the second relapsed after 3 months; while the third was still negative after 5 months. Three of the other cases that showed improvement relapsed, however, during the first week of treatment and so were considered failures.

The second group, comprising 6 cases of acute dysentery were given fumagillin in the above dose together with 4 gm. of neomycin daily for a period of 8 days. Four of these 6 cases showed moderate improvement in the clinical symptoms within a period of 3–5 days, while the *Entamoeba* disappeared from the stools on the third day in three, and on the fifth day in the fourth. Two of these did not relapse within one month of observation, while the two other cases relapsed 21 days and 3 months later. Two cases of the group did not improve until the 7th day and the 12th day respectively; both complained of a complicating bilharzial dysentery, but they were not followed up.

The third group was composed of 4 patients, 3 of whom were given a daily dose of 4 grams of neomycin, while the fourth was given 6 gm. daily; the course of treatment was usually 6 days. Three of the four cases did not show any clinical or parasitologic response. The stools remained positive for amebae during a period of 2 weeks observation.

The fourth group included 6 cases given fumagillin in a daily dose of 200 mg. for ten days. In all of these recurrent cases, marked clinical improvement occurred in 3–4 days' time, while the *Entamoeba* disappeared in a similar period. They remained negative for two months after the end of the course and no clinical or parasitic relapses have occurred so far. Routine liver and kidney function tests did not indicate any toxic effect of the drug. No side effects developed in these cases.

DISCUSSION

The trial of fumagillin in amebiasis is interesting because it is the only antibiotic so far discovered which has no antibacterial action and is claimed to be a most potent amebicidal drug. This work did not show the promising results reported by Killough *et al.* (1952) and Anderson (1952). The discrepancy between our results and theirs is possibly due to the fact that our cases were suffering from acute dysentery while their group was formed of chronic cyst carriers.

Moreover our patients represent severe relapsing cases that had resisted other lines of treatment and were usually complicated by the association of bilharzial dysentery. These conditions would delay the clinical response to any particular drug, slow the recovery and predispose to more frequent relapses. This was our experience in most of the cases given either emetine or antibiotics. The use of fumagillin in a dose of 200 mg. daily appears however to be more promising, as all the cases improved rapidly in 3–4 days' time, while *Entamoeba histolytica* disappeared from the stool in a similar time. The 6 cases given this larger dose were observed for two months and so far no clinical or parasitic relapse has occurred. It is intended in the future to use these doses or give slightly higher doses provided no toxic symptoms arise.

In the doses so far used, no toxic symptoms or evidence of liver or kidney impairment was observed. It was reported by Hrenoff *et al.* (1951) that doses of 125 mg. per kilogram given to monkeys caused slight liver impairment as demonstrated by bromsulphalein test.

Neomycin which was used in 4 cases of acute amoebic dysentery did not prove encouraging. The result of the use of a combination of neomycin and fumagillin gave better results, possibly because of the synergistic effects of the drugs, the former having marked bacteriostatic action, while the latter is an active amebicidal drug.

ADDENDUM

Fumidil®, another brand of fumagillin, was kindly supplied by Abbott Laboratories. In order to evaluate the effectiveness of this drug in comparison with fumagillin it was given to a group of ten cases of acute amebic dysentery. The same scheme of investigation and follow-up was followed as previously described, while the daily dose used was 60 mg. given for a period of 10 days. In all these cases, the clinical symptoms rapidly improved within a period of 5–8 days, while *E. histolytica* was not found in the stools of the patients within a similar period. In 3 cases however the diarrhea persisted in a mild form for a few days after the end of treatment. One case developed some form of erythema all over the body on the 4th day of treatment, which disappeared in three days' time.

The activity of Fumidil against intestinal amebiasis has thus been demonstrated in this small series of cases. The non-activity of similar brands of fumagillin may be due to the fact that the stocks used were not refrigerated, which may account for their loss of potency especially if kept for several months.

Fumidil was found of especial value in the treatment of chronic cyst carriers

whether healthy or suffering from chronic diarrhea. This agrees with the experience of Anderson *et al.* (1952) in America and Killough *et al.* (1952) in Egypt. The same course used in acute cases is to be followed in chronic cases. It is interesting to find that the doses recommended by these authors did not exceed a total dosage of 100 mg. given over a period of 12½ days. Anderson, however, used in monkeys a much higher dose approaching 250–650 mg. per kilogram daily and found lesser doses ineffective.

With regard to the synergistic action of antibiotics it was recently reported by Anderson *et al.* (1954) that the combination of erythromycin and fumagillin had a synergistic action if used in the treatment of amebic infection in monkeys. Erythromycin alone was found useful in the treatment of amebiasis by Nor El-Din (1956). Oxytetracycline and fumagillin were tried together in a dose of one gram of the former with 60 mg. of the latter. In my experience this combination is on the whole better than the use of either alone, but the follow-up of these cases is not yet completed. On the other hand I would suggest the combination of oxytetracycline with erythromycin in the treatment of amebic infection because this was found promising. This combination is quite logical as the former drug is bacteriostatic, while the latter is directly amebicidal. This combination is useful also because it may prevent the complication of staphylococcal enteritis which may develop after the use of oxytetracycline alone.

The mode of action of the different antibiotics in the treatment of amebiasis is a matter of interest. In many instances the results of in vitro work did not correspond to the in vivo observation. This is best exemplified in the case of fumagillin which is very active in vitro but its result in man is conflicting and not quite satisfactory. The fact that fumagillin, which is a direct amebicide, is less satisfactory than oxytetracycline favor the suggestion that the antibiotics affect the ameba in the lumen of the gut by depriving it of food bacteria and not by direct action. The presence of intestinal bacteria will help the ameba to survive because it causes diminution in the oxidation reduction potential; the use of the antibiotics causes a shift to a more positive potential which is lethal to the ameba. The study of the metabolism of the ameba in culture under special conditions, and the effect of various drugs on its metabolism, is a recent and scientific method of drug evaluation in the treatment of amebiasis.

SUMMARY

Fumagillin, a new antibiotic, was tried in daily doses varying from 60–200 mg. in the treatment of acute amebic dysentery. The course was given usually for a period of 8–10 days. In the maximum daily dose of 200 mg., the drug was found effective in the symptomatic cure of the disease, while the *Entamoeba* usually disappeared from the stools 3–6 days after the start of treatment. Doses smaller than 100–150 mg. daily were inefficient.

Fumagillin was found however less effective than oxytetracycline or chlortetracycline, while the relapse rate after treatment was higher. Fumidil®, another drug of the same structure as fumagillin, was found more effective than fumagillin even when given in smaller doses. A dose of 60 mg. daily for a 10 day course

was almost equal to 200 mg. daily dose of fumagillin given for a similar period. It is probable that Fumidil as used in this work was found more potent than fumagillin because the latter lost its potency by storage before use. Refrigeration is required to retain potency.

Side effects after both drugs are uncommon but include skin rashes and dermatitis, possibly of allergic origin.

Neomycin, another antibiotic, was found ineffective in the treatment of acute amebiasis when used in a daily dose of 4 grams for a period of 6 days. Its addition to fumagillin proved slightly better than either drug used alone.

The trial of a combination of fumagillin with oxytetracycline was found more promising and will be continued.

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ERYTHROMYCIN IN THE TREATMENT OF AMEBIASIS

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During recent years the introduction of antibiotics in the therapy of amebiasis marked a great advance in this field. Most antibiotics have been used at one time or another with varying success. Chlortetracycline (Aureomycin) and oxytetracycline (Terramycin) have been used in Egypt by Halawani (1951) and by the author (1954) in the treatment of amebiasis with encouraging results, although relapses usually occurred in about one third of the cases. Chloromycetin and neomycin have been used by the author (1954), with slightly better results in the case of the former. However, neither drug has proved encouraging in treatment, as the dysenteric symptoms usually continue for a period of at least seven to ten days after the start of treatment. In some few cases however the dysenteric symptoms were moderately ameliorated but *Entamoeba histolytica* usually persisted during the period of observation which extended in some cases to about one month. Penicillin and oral streptomycin were used by my colleagues and myself in the Cairo Research Institute but the results were not encouraging.

Fumagillin was used on a large scale in Iraq and in Egypt and showed marked activity in vivo when tested on amebic infection in monkeys, and in doses of 200 mg. daily proved to be of some value in human amebiasis; its effect, however, was considered inferior to that of chlortetracycline or oxytetracycline.

A new antibiotic, erythromycin, has been introduced recently, produced from a strain of *Streptomyces erythreus* isolated from the soil of the Philippines. This antibiotic is indicated in infections caused by gram positive bacteria especially those resistant to penicillin, and is claimed to affect the large viruses and some protozoa. The recent work of McCowen (1953) suggests its effect on *E. histolytica* in vitro and in vivo. The drug is moderately absorbed from the intestine especially if given on an empty stomach before food, and it is claimed that a special coating of the tablets facilitates its absorption in the blood. The drug is excreted in the feces in large quantities which may suggest its value in intestinal infections.

METHODS

Erythromycin in the form of Ilotycin (Lilly) or Erythrocin (Abbott) was given to a group of 15 adult patients suffering from acute amebic dysentery. Ten patients were given the drug in a dose of three 100 mg. tablets every 6 hours which means 12 tablets or 1.2 grams daily. This dose was given for a period of ten days. A smaller group of patients were given the drug in the same dosage for a period of only 5 days. A daily stool examination for the presence of *E. histolytica* was done during a period of one month in hospital. The daily number and the character of stools was accurately recorded, sigmoidoscopy was done on most of the cases before and after treatment. Routine hematologic examination was done to reveal any toxic effect of the drug on the bone marrow, while liver

and kidney function tests were routinely done in most cases. Daily blood pressure determinations were done since some references in the literature suggested that toxic doses of the drug in dogs might cause lowering of the blood pressure.

RESULTS

In almost all the cases the dysenteric symptoms rapidly improved, and within a period of 3–4 days spectacular results were evident. In some cases the daily number of stools decreased from an average of 15–20 to 3–5 in this brief period. *E. histolytica* usually disappeared from the stools of the patients in a similarly short time. In some few cases (4 patients) however, the *E. histolytica* vegetative forms rapidly disappeared from the stool to give place to cysts. In these latter cases the improvement in the symptoms was gradual and incomplete. In a follow up of some of these cases, however, the amebic cysts disappeared from the stool in an average time of seven days after the start of the treatment.

PROGRESS OF THE CASES

A follow up for an average period of three months was done. Of the fifteen cases given treatment, two cases relapsed, both of them ten days after the start of treatment. They both suffered from the recurrence of diarrhea and the reappearance of *E. histolytica* in the stool. These two cases were in the group of five patients given the shorter five-days course of treatment. In the other group of ten patients given the ten days course no relapse had occurred three months after the course of treatment was given. Sigmoidoscopic examination revealed the absence of active intestinal ulcers in the cured cases, while few ulcers were seen in the two relapsing cases.

It is noteworthy that no side effects occurred in the patients during the course of therapy, although some cases complained of mild nausea and anorexia; a few complained of being rather drowsy, while others complained of general pains all over the body with a feeling of lassitude.

TRIAL OF ERYTHROMYCIN IN DIARRHEA DUE TO GIARDIA LAMBLIA INFECTION

The drug was tried in a severe case of acute dysentery due to heavy infection with *Giardia intestinalis*. The patient was given the usual course of atabrine without showing any amelioration of the symptoms. When a course of Ilotycin was given in the same dosage as that given in the amebic dysentery cases, rapid improvement in the clinical symptoms occurred in 2–3 days time, while the Giardia parasite disappeared from the stool of the patient in a similar time. The drug was then used in two more cases of *Giardia lamblia* infection with the same encouraging results.

DISCUSSION

The results of the present work suggest that erythromycin can be useful in the treatment of amebiasis and confirms the previous work of McCowen (1953). According to this author erythromycin was found more active than chlortetracycline or oxytetracycline in the treatment of intestinal amebiasis in rats. The

action of erythromycin on the intestinal ameba seems to be more direct; it thus differs from most other antibiotics which affect the intestinal ameba by indirect action through inhibition of the associated intestinal bacteria. Erythromycin had no action on *Entamoeba coli* in the intestine, as was proved by its effect on the count of *E. coli* in the stool of a patient given this drug.

The role of the intestinal bacteria especially of *Escherichia coli* on the pathogenesis of amebiasis has been referred to by various authors. Erythromycin does inhibit some anaerobic organisms like clostridia and other gram positive enterococci like *Streptococcus faecalis* and some strains of staphylococci. Vogel (1945) has referred to the importance of some enterococci in the pathogenesis of amebic dysentery. Erythromycin may be useful then in the treatment of intestinal amebiasis by affecting these organisms if they have a definite role in the pathogenesis of the disease.

However, it seems from the results of the in vitro work that the drug has a high direct amebicidal effect. In this respect this drug is similar to fumagillin although the latter drug did not prove as promising as was claimed at first. Erythromycin has the advantage over other antibiotics like oxytetracycline in that it does not affect the bacterial flora of the intestine. This is an advantage because no side effects such as symptoms of vitamin deficiency or monilial infections follow its use as reported after the wide-spectrum antibiotics. Moreover it has been reported that some cases developed severe diarrhea during the course of oxytetracycline therapy, due it was supposed to a change of the bacterial flora. Some strains of staphylococci flourished, causing enteritis and subsequent diarrhea. This complication has not so far been reported in my series or referred to in the available literature.

The course suggested in the treatment of acute intestinal amebiasis is a ten day course consisting of a daily dose of 20 mg. per kg. body weight of the drug. In the average adult of 60 kg. body weight the daily dose is about 1.2 to 1.5 gm. of the drug. The results of the present work suggest that a course of 5 days at the same daily dose is inadequate, as relapses occurred in two out of 5 cases given this short course.

The absence of side effects after the use of erythromycin is quite striking. The liver and the kidney function tests did not show any toxic effect of the drug. No effect on the bone marrow was observed.

Chloramphenicol and neomycin which have been used in the treatment of amebiasis have not proved to be safe drugs. The former usually affects the bone marrow and may lead to a plastic anemia, while the latter may cause toxic reaction in the kidney. Studies on the toxicity of erythromycin in animals have confirmed its low toxicity.

Some transient lowering of the blood pressure has been reported in dogs following the intravenous administration of erythromycin. This complication was not seen amongst my patients given the oral treatment.

The fact that erythromycin is concentrated in the liver, as found by Heilman (1952), is a point of interest as this may suggest its possible value in the treatment of amebic hepatitis, and we expect soon to investigate this. The trial of

erythrocin in combination with oxytetracycline has also so far proved more encouraging than either antibiotic used alone.

SUMMARY

Erythromycin was found of value in the treatment of acute amebic dysentery. The drug was given to ten cases in a dose of 1.2 gm. daily for a period of 10 days and in a similar dose to another group for a period of 5 days. Marked improvement appeared in both groups, but two cases relapsed in the second group, while no relapses occurred in the first. The follow up is not complete however, as it was continued only for an average of 3 months. It is suggested that further trials be made, with a longer period of observation.

ADDENDUM

Recently a new drug, carbomycin (Magnamycin, Pfizer), similar to erythromycin, has appeared, prepared from *Streptomycin halstedii*. It seems to have a similar chemical constitution to erythromycin. We have tried it in 6 cases of acute amebic dysentery with the same encouraging results. This drug is now under trial in combination with oxytetracycline, with promising results.

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WEST NILE VIRUS INFECTION IN ARTHROPODS[1]

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The work reported here, limited to the experimental infection in arthropods, is a special phase of a broad study of the ecology of West Nile virus in Egypt. A comprehensive report of this study including a review of the literature will be found in another paper (Taylor *et al.*, 1956).

Philip and Smadel (1943) were the first to show that West Nile virus could be transmitted by a mosquito. It was subsequently isolated from naturally infected mosquitoes in a highly endemic area in the Nile Delta of Egypt (Taylor and Hurlbut, 1953). Additional observations on the experimental transmission of the virus by mosquitoes have been reported by Kitaoka (1950) and Davies and Yoshpe-Purer (1954).

MATERIAL AND METHODS

Except where indicated in Table 1, virus strain Ar-248 was used throughout these experiments. This strain was isolated from *Culex* mosquitoes in the Nile Delta in the summer of 1952 (Taylor and Hurlbut, 1953). It was initially carried through three mouse brain passages and then maintained in a manner simulating natural transmission by mosquito-mouse cyclic passage. Strain E101, isolated from human serum in Egypt by Melnick *et al.* (1951), was used in the ninth to the twelfth mouse brain passage, in a few tests.

All of the arthropods were collected in the Nile Delta except the rat flea *Xenopsylla cheopis*, which was from a laboratory colony of undetermined origin. *Culex pipiens, C. antennatus, C. univittatus, Musca domestica vicina, Ornithodoros savignyi, Argas persicus, Pseudolynchia canariensis, Periplaneta americana*, and *Blatella germanica* were reared in the laboratory; the remaining arthropods, as listed in Table 1, were collected in the field.

The virus was introduced parenterally either by the puncture method (Hurlbut, 1951) or by injecting 0.001 ml. of the virus preparation into the hemocoele. In the puncture method, the body wall was simply punctured by a steel needle which had been dipped in 10 per cent infected mouse brain suspension. The steel needle had a diameter of 0.1 mm. with a well tapered point. The injections were made with a glass needle from tubing having an outside diameter of 1.0 mm. and a point diameter of 0.085 mm. The needle was mounted on a one quarter ml. tuberculin syringe by means of a special chuck. The syringe was

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The opinions or assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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TABLE 1

Growth spectrum in arthropods for West Nile virus after parenteral introduction. (Virus concentrations based on total body weight. When negative, the lowest dilution is given. Original inoculum 10 per cent infected mouse brain. Virus introduced by puncture except as noted)

Arthropod group	Species	Titer inoculum	Titer arthropod 1st post-inoc. day	Further incub. days, 8–32°C	Titer arthropod
Mosquitoes	Culex pipiens L.	6.8	3.0	4	6.2
	C. antennatus (Becker)	3.0*	NT	27	7.2
	C. univittatus Theobald	7.9	3.0	6	7.9
	Culiseta longiareolata (Macquart)	8.3	NT	3	>6.4†
	Aedes caspius Pallas	9.2	3.4	4	5.9
House flies	Musca domestica vicina Macq.	8.7	Neg. 1.0	10	4.2
Soft ticks	Ornithodoros savignyi (Audouin)	8.8	Neg. 1.0	11	>4.0†
	O. erraticus (Lucas)	7.7*	NT	27	5.3
	Argas persicus (Oken)	9.2	NT	6	2.2
	A. persicus	5.7*	NT	24	Neg. 1.7
Hard tick	Rhipicephalus sanguineus Latreille	7.9	NT	14	>5.7
Lice	Pediculus humanus corporis Deg.	8.8	Neg. 2.0	3	3.6†
Mites	Dermanyssus gallinae (L.)	5.4*	NT	16	Neg.†
Bedbugs	Cimex lectularius L.	8.4	NT	8.19	Neg. 1.7†
Fleas	Xenopsylla cheopis (Roths.)	7.9	NT	14	Neg. 2.2
	X. cheopis	>7.0*	NT	14	Neg. 1.9
Hippoboscid flies	Pseudolynchia canariensis (Macquart)	5.3*	NT	14	Neg. 1.0
Cockroaches	Periplaneta americana (L.)	7.9‡	Neg. 1.0	19	Neg. 1.0
	Blatella germanica (L.)	8.8‡	Neg. 1.0	7	Neg. 1.0†

* Titer of blood. Infected by blood feeding. NT = No test.

† Strain E.101; all others Ar-248, 3rd mouse brain passage plus 1–7 mosquito-mouse cyclic passages.

‡ Virus introduced by injecting .001 ml. into hemocoele.

held in a modified Krogh Key pipette with the screw mechanism calibrated to deliver 0.001 ml. The syringe was kept partly filled with liquid petrolatum and the amount of virus suspension to be inoculated was drawn into the point of the needle and expelled with each inoculation. An air space was maintained between the liquid petrolatum and the virus suspension. The apparatus was held in the hand and the carbon dioxide-anesthetized arthropod simply impaled through the thoracic wall during the injection. It was found convenient to fill the needle from a drop of the virus suspension which was placed on a glass slide having a thin coating of paraffin.

Glass fruit jars of one quart size were used as containers for infected mosquitoes. The top was covered with cloth netting held on by rubber bands. A 9 mm. circular hole in the netting, supported by tape, served for introducing or

withdrawing mosquitoes. The hole was closed when not in use by inserting a glass feeding tube 8 mm. in diameter having a 20 mm. bulb blown in the upper end. The tube was filled with 5 per cent dextrose. A chick or suckling mouse was immobilized on the netting for blood feeding.

Titrations of the virus content of arthropods was accomplished by triturating the whole body, and injecting intracerebrally into adult mice the centrifuged supernatant in appropriate dilutions based on the total body weight of the arthropod. The LD_{50} was estimated according to the method of Reed and Muench and expressed as the logarithm of the dilution.

POTENTIAL ARTHROPOD HOST RANGE

In the present study, as a preliminary step, representative arthropods were tested for parenteral susceptibility by injecting the virus into the hemocoele. It seemed obvious that natural hosts would be found only among those arthropods in which the virus could propagate, and it was assumed that the parenteral route was the most sensitive as well as the most convenient. The potential hosts thus determined were given further study, as will be indicated later. It was believed also, that the range of parenteral susceptibility in arthropods might prove to be useful in the systematic classification of arthropod-borne viruses.

The results of this preliminary survey are given in Table 1. Titrations of the arthropods were done in representative groups 24 hours after inoculation, and again after incubation for a longer period in order to determine whether an increase in titer had taken place. In most cases the volume of inoculum was not determined since the puncture method was used. In a few instances the virus was introduced by blood feeding. Mosquitoes attained the highest virus titer of all the arthropod groups tested. The virus was also propagated without difficulty in adult houseflies. We failed to recover it from the salivary secretion of flies, and infant mice did not become infected when these insects fed on areas of scarified skin. The hippoboscid fly *Pseudolynchia canariensis*, a pigeon parasite, failed to become infected when it fed upon an infected chick. Parenteral infection was not tried. The human body louse apparently became infected when the virus was introduced by puncture. This experiment has not been repeated. We have no evidence that the louse is involved in the epidemiology of the disease. There was evidently no propagation in the bedbug, the rat flea, or the cockroach following parenteral introduction. Chicken mites were refractory in a single test when they were fed infected blood. Both soft and hard ticks were quite susceptible to infection and, in addition to the mosquitoes, this group was selected as the most promising for further investigation. The results with mosquitoes and ticks will now be discussed in more detail.

EXPERIMENTS WITH MOSQUITOES

The virus multiplies very rapidly in mosquitoes at 28–32°C., as will be seen in Table 2. On the first postinoculation day the titer was 3.0, on the second day 6.2, and it probably reached its maximum of between 7 and 8 on the fourth day, remaining at this level with little change for 17 days. Between the 21st and 36th

TABLE 2

West Nile virus titer in Culex univittatus *1 to 48 days after inoculation 28–32°C. Pools of 5 mosquitoes*

Post inoculation day	Titer	Transmission by bite
1	3.0	
2	6.2	1/3
3	6.2	3/3
4	>7.6	3/3
5	7.1	2/2
6	7.9	
7	7.3	1/1
11	7.2	
14	7.0	5/5
21	7.4	
36	6.1	
42	6.1	
Single mosquitoes, last survivors 48th day	6.1	
	6.6	
	4.9	
	5.9	

TABLE 3

West Nile virus titer in Culex univittatus *after feeding on infected mouse blood. Incubation at 28–32°C. Blood titer, 5.8*

Days after infective feeding	Titer	Transmission by bite*
1	4.2	0/4
2	6.2	0/6
3	6.6	
4	7.3	0/2
5		2/2
6	7.4	1/1
7		1/1
8	7.5	
12	7.4	
28	6.5	

* Another group similarly treated.

day, a drop of about one log took place; however, virus was present in relatively high titer even after 48 days. It was transmitted by bite to one out of three mice bitten on the second postinoculation day and to all of 14 mice bitten between the third and thirty-sixth day. A similar pattern of virus titers was observed when the agent was ingested in the course of normal blood feeding, with the probable difference that the initial rise is somewhat less rapid. The virus was transmitted by bite as early as the fifth day after the infective feeding and as late as the 33rd day. The data are presented in Table 3. Virus was not recovered from the offspring of infected females.

TABLE 4

Serial passage of West Nile virus in Culex univittatus *by puncture. 5 mosquitoes pooled for each titration*

Passage	Days incubation at 28–32°C	Titer
1*	4	7.1
2	7	8.0
3	12	7.9
4	5	7.6
5	7	6.4
6	7	7.6
7†	10	8.1

* Dilution $10^{-1.6}$. Original inoculum 10 per cent mouse brain, titer 7.9.

† 4/4 mice bitten by mosquitoes of passage 7 became infected.

TABLE 5

Blood titer in mice after 24 hours incubation. Infection induced by mosquito bite

Age mouse days	Blood titer
5	7.6
6	6.2
6	4.4
16	3.6
18	4.6
31	3.4
36	2.5
42	2.4
44	1.6

TABLE 6

Susceptibility of Culex antennatus* *to infection with West Nile virus induced by ingestion of infected mouse blood. (Incub. 14 days at 28–32°C)*

Blood titer	No. mosquitoes† infected	No. not infected
1.6	1	1
2.4	4	10
2.5	13	0
2.6	4	0
2.8	15	0
3.6	5	0
4.3	10	0
4.5	19	0
4.6	13	0

* Collected in the field as larvae, or from eggs deposited in the laboratory by wild females.

† Triturated individually and injected into 5 mice.

In another test, virus was propagated readily through seven serial passages in mosquitoes which were kept on a diet of dextrose, no blood being given at any time. The titer of the seventh passage was 8.1 and all of four mice bitten by these mosquitoes became infected. The data are presented in Table 4.

TABLE 7

Susceptibility of Culex univittatus* *to infection with West Nile virus induced by ingestion of infected mouse blood. (Incub. 14 days at 28–32°C)*

Blood titer	No. mosquitoes infected	No. mosquitoes not infected
1.5	1	8
2.0	4	7
2.4	7	4
2.5	4	6
2.6	3	6
3.4	3	5
3.6	10	0
4.2	10	0
4.6	28	0

* Laboratory colony.

TABLE 8

Susceptibility of Culex pipiens* *to infection with West Nile virus induced by ingestion of infected mouse blood. (Incub. 14 days at 28–32°C)*

Blood titer	No. mosquitoes infected	No. mosquitoes not infected
2.2	3	6
2.6	2	9
3.6	4	2
4.5	7	0
6.2	15	0

* Laboratory colony.

The virus titer attained in the blood of mice decreased progressively with the mouse age between 5 and 45 days. This furnished a gradient (Table 5) which was utilized in testing the susceptibility of mosquitoes to infection through normal feeding on infected blood. The mosquitoes were triturated individually after incubation for 14 days at 28–32°C., and the supernatant of the centrifuged suspension was injected into five mice. Three species were tested in this manner (Tables 6, 7, 8). The results show considerable variation, but as a rough generalization it may be said that a titer of 2.5 will probably infect about 50 per cent of *C. antennatus* and *C. univittatus*. *C. pipiens* seems to require a somewhat higher concentration. The first two species have been found infected in nature. The frequency of infection in nature is consistently higher in *C. univittatus* (Taylor *et al.* 1956). The data indicate that a titer of 4.5 may be expected to infect practically all individuals of these three species. Further work using wild birds which are known to harbor natural infections of West Nile virus is reported elsewhere (Work *et al.*, 1953; 1955).

Parenteral susceptibility of *C. univittatus* to infected blood was tested by injecting 0.001 ml. of serially diluted serum into the hemocoele. The mosquitoes were subsequently triturated individually and five adult mice were injected with the centrifuged supernatant. Compared with adult mouse intracerebral titrations using 0.03 ml., the limiting dilutions were found to be approximately

TABLE 9

Susceptibility of Culex univittatus *to parenteral infection with West Nile virus. Serial decimal dilutions of infected mouse serum injected into the hemocoele*

Test	Log dilution						ID_{50}
	4	5	6	7	8	9	
1. Mosquito control, mouse, ic	5/5	4/5	1/5	0/5			5.50
	5/5	5/5	3/5	0/5			6.17
2. Mosquito control, mouse, ic			4/5	2/5	0/5	0/5	6.68
			5/5	2/5	0/5	0/5	6.75

* Volume of inoculum: Mosquito 0.001 ml.; Mouse 0.03 ml.
Extrinsic incubation: 10 days at 28–32°.

TABLE 10

Transmission of West Nile virus by mosquitoes at outdoor temperatures, Cairo, Egypt. February–April, 1954

Blood titer	Incub. days	Temperature* daily mean		Mosquitoes infected		Mice infected by bite	
		Max.	Min.	No. tested	No. infected	No. bitten	No. infected
Culex pipiens							
		°C	°C				
3.8	28–33	22	13	5	5	4	1
6.6	15–16	23	13			2	1
	18–29	26	14	5	5	2	1
Culex univittatus							
6.6	15–16	23	13			2	0
	26–29	26	14	4	4	1	1

* Absolute range: 33–9°C.

the same. Assuming that an intracerebral infective dose is equivalent to a lethal dose in a mouse, and adjusting for the difference in the volume of the inoculum, it appears to require somewhat less virus to infect a mosquito, the difference being of the order of one log. The data are presented in Table 9. Comparing the parenteral route with the normal ingestion of infected blood, the latter appears to require about 30 mouse LD_{50} in *C. univittatus*, based upon an average blood meal of 0.003 ml. Thus the parenteral route is probably some 300 times more sensitive.

In all the foregoing tests the mosquitoes were incubated at approximately 30°C. However, the virus may propagate in the mosquito and be transmitted at mean daily temperatures of 12–23°C. with an absolute range of 9–33°C. This was demonstrated by placing the mosquitoes outdoors at these temperatures, permitting them to bite infected mice, holding them for 15–33 days at these same

low temperatures, and then allowing them to bite normal mice (Table 10). It is thus experimentally possible for this agent to be transmitted throughout the winter months in Egypt. *C. pipiens* is known to take blood and breed throughout this season of the year.

EXPERIMENTS WITH TICKS

It has been demonstrated that the tick *O. savignyi* can be infected with the virus and retain it for at least 87 days. Specimens infected parenterally may transmit it while taking blood from infant mice (Taylor and Hurlbut, 1953). Further tests showed that the coxal fluid secreted after engorgement by parenterally infected ticks, contained the virus (Table 11). Similar transmission tests with *Ornithodoros erraticus* and *A. persicus* have not been successful, although in one instance the virus was recovered from the coxal fluid of *A. persicus*. Transmission tests with these ticks when infected by feeding have also been unsuccessful. The data are summarized in Table 12.

TABLE 11

Transmission of West Nile virus by Ornithodoros savignyi. *Ticks infected by puncture*

Incub. days 30°C	No. ticks taking blood	Virus in coxal fluid*	Vertebrate host bitten	No. vert. infected by bite†
14	4, 6	Not tested	mouse	2/2
15	7, 8	5/5, 5/5	mouse	2/2
15	8	1/5	mouse	0/1
30	6	5/5, 2/5, 0/5		
		4/5, 2/5, 0/5	sheep	0/1

* Coxal fluid pooled from ticks on each mouse and injected into 5 mice. Fluid from each tick fed on sheep tested separately by injecting into 5 mice.

† Confirmed by bacteria free passage.

TABLE 12

Ticks infected with West Nile virus by ingesting infected blood

Ticks	Blood titer	Incub. days	Ticks titer	Transmission test	
				No. ticks taking blood	Mice infected by bite
Ornithodoros erraticus	7.8	11–21	—	6	0/3
		30	4.7		
	7.6	10–28	—	15	0/6
	7.7	8		2	0/1
		27	5.3		
	5.8	14		8	0/2
O. savignyi	5.5	21	—	6	0/2
		33	3.5		
Argas persicus	5.7	26	Neg.		
	6.3	11, 31		20	0/2 chicks

DISCUSSION

It is apparent from the foregoing that certain mosquitoes are highly susceptible to this Egyptian strain of West Nile virus (Ar-248), being readily infected by circulating titers well within the range to be expected in avian or human infections (Work *et al.*, 1955; Southam and Moore, 1952). The infection develops rapidly in these mosquitoes and their bites become infective within five days at 30°C. It is possible, therefore, that the virus may be transmitted during the second gonotrophic cycle in the summer months, since eggs are deposited three or four days after the first blood meal. The gonotrophic cycle is the reproductive cycle starting with the taking of blood and ending with the depositing of eggs, which is repeated every three or four days throughout the life of the mosquito. The virus will also propagate in mosquitoes at temperatures prevailing in Egypt during the winter months, and mosquitoes can transmit the agent by bite under these conditions. Thus, it is possible that *C. pipiens*, which is active in and around human dwellings at this time of year, may transmit the virus.

The susceptibility of long-lived arthropods such as ticks suggests that they may constitute a reservoir. However, there is no epidemiological evidence to support this hypothesis at the present time. From another point of view, the arthropod host base may be broader than is visualized at present. One ordinarily thinks of these infections in arthropods as being acquired through ingested blood from an infected vertebrate. However, in view of the relatively wide range of parenteral susceptibility, the possibility of parenteral introduction by parasites of the arthropods should, perhaps, not be overlooked.

SUMMARY

1. Mosquitoes, ticks, houseflies, and probably human body lice were found to be susceptible to parenteral infection with West Nile virus. Chicken mites, bedbugs, rat fleas, hippoboscid flies, and cockroaches proved to be refractory.

2. *Culex univittatus*, *C. antennatus*, and *C. pipiens* were infected readily when blood having a virus titer of 4.5 was ingested. A titer of 2.5 was sufficient to infect many individuals.

3. *C. pipiens* and *C. univittatus* became infected and were capable of transmitting the virus when kept at mean daily temperatures of 12–23°C.

4. *C. univittatus* transmitted the virus as soon as 5 days after the infective blood meal and for at least 33 days thereafter at 28–32°C.

5. The tick *Ornithodoros savignyi*, when infected parenterally, transmitted the virus while biting infant mice, and the virus was isolated from the coxal fluid. This tick was also infected by feeding on the blood of infected mice, but failed to transmit the virus when infected in this manner. *Ornithodoros erraticus* and *Argas persicus* showed evidence of infection but did not transmit the virus.

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THE TRANSMISSION OF WESTERN EQUINE ENCEPHALITIS VIRUS BY THE MOSQUITO *CULEX TARSALIS* COQ[1, 2]

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Numerous epidemiological investigations have indicated that Western equine encephalitis is a mosquito-borne disease. This was further established and elaborated by evidence incriminating the mosquito *Culex tarsalis* as the important vector in the western part of the United States. The virus was first isolated by Hammon *et al.* (1941) from wild-caught *C. tarsalis* in the Yakima Valley of Washington and since that time numerous isolations of virus have been reported from this mosquito in a wide area of the western United States and Canada. Reeves (1953) reports that from California alone, 177 isolations of virus from *C. tarsalis* have been obtained. Occasional isolations of virus have been reported from other species of mosquitoes, but investigations in the field have not indicated any of these species to be of importance in the natural dissemination of the disease. The only reported studies in the laboratory on the transmission of Western equine encephalitis virus by *C. tarsalis* were made by Hammon and Reeves (1943, 1946). They first reported twelve transmissions, infecting mosquitoes by permitting them to feed on an inoculated guinea pig, on an inoculated duck, and on virus-blood suspensions. These mosquitoes then transmitted the virus to chickens and to a guinea pig through their bites. Subsequently (1946) transmission was effected twice by mosquitoes infected by feeding on inoculated chickens but the details of these and other transmissions were not published.

Studies by Reeves and Hammon (1944), and Hammon *et al.* (1945–1945a) have indicated that wild and domestic birds are the preferred hosts of *C. tarsalis*. However, Reeves and Hammon (op. cit.) found that *C. tarsalis* will also feed on livestock and man, and Brennan (personal communication) states that in the summer of 1952 this species was the chief mosquito pest of man in Bakersfield, California. Further, Hubert, Rush, and Brennan (1954) were able to rear eight generations of this mosquito in the laboratory on nothing but human blood.

Evidence from surveys in the field and investigations in the laboratory has established that wild and domestic birds also are involved in the epidemiology of the disease. Neutralizing antibodies have been detected readily in both wild and domestic birds. Further, Hammon and Reeves (1946) demonstrated that

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viremia could be induced regularly in chickens by subcutaneous inoculations of virus, and Hammon *et al.* (1951) also demonstrated high grade viremias in four species of wild birds following subcutaneous inoculation of virus. Evidence of the involvement of birds in the Western equine encephalitis cycle is so strong that it is believed now that avian species are the primary vertebrate hosts of the virus.

It has been evident that further detailed studies on the transmission of Western equine encephalitis virus in birds by *C. tarsalis* were needed and the colonization of this mosquito at the Rocky Mountain Laboratory by Brennan and Harwood (1953) provided a unique opportunity to undertake such studies.

MATERIALS AND TECHNIQUES

The strain of Western equine encephalitis virus used in this work was isolated by Dr. Carl M. Eklund from a pool of 25 female *Culex tarsalis* collected by Dr. James M. Brennan in Bakersfield, California, during the last half of June, 1952. The stock virus used in the work had been prepared after a total of two passages through suckling white mice as a 10^{-1} suspension of mouse brain in 10 per cent normal inactivated rabbit serum and 90 per cent isotonic saline. This stock virus was then sealed, frozen in ampules, and held at $-70°C$.

Both chicks and canaries were used to infect mosquitoes in studies of transmission. Canaries were used more often than chicks. Chicks were usually two to four weeks old when inoculated but the age of canaries was unknown. Birds were infected with virus by subcutaneous inoculation of 0.05 cc. of a 10^{-5} dilution of stock virus.

In attempts at transmission of virus through mosquitoes, birds were inoculated with virus as indicated above; then, after 20 to 24 hours, blood was drawn from the infected bird for titration, after which the infected bird was placed in a cage with normal mosquitoes for periods varying from a few minutes to eight hours. Chicks were used almost exclusively as the normal recipient host in attempts at transmission and they were placed with the test mosquitoes for periods varying from a few minutes to several hours. Approximately 24 hours after mosquitoes had fed on such birds, blood was drawn from the bird, a 10^{-1} dilution of the blood was prepared in 10 per cent normal inactivated rabbit serum-saline, and a litter of 4-day-old suckling mice was inoculated intraperitoneally with 0.05 cc. of this whole blood dilution per mouse. During the first half of this study, seven mice per litter were used, but because a shortage of mice developed later, this number was reduced to six during the latter half of the work.

Six mice per litter were used in all titrations of virus. All mice were routinely observed for seven days after the day of inoculation. All inoculations were made intraperitoneally, using 0.05 cc. of inoculum. For the identification of viral agents recovered after transmission, antigens for neutralization tests were prepared as 10 per cent suspensions of mouse brain in 10 per cent normal rabbit serum-saline. However, when brains were taken from dead mice, 300 units of penicillin and 300 micro-grams of streptomycin were added to each milliliter of diluent.

Chicks and white mice were reared at the Rocky Mountain Laboratory under

conditions which excluded the possibility of previous infection with virus. Canaries were obtained from a large pet supply house in St. Louis, Missouri, and all were found to be susceptible to primary infection with Western equine encephalitis virus.

The methods employed at the Rocky Mountain Laboratory for the rearing of *C. tarsalis* have been described in detail by Brennan and Harwood (*op. cit.*) and by Hubert *et al.* (1954). Rearing was initiated with eggs taken at intervals from the main colony. All work with mosquitoes, including rearing, transmission, and maintenance of infected stocks of mosquitoes, was done in a room in which the temperature was maintained at 24°C. (75°F.) and the relative humidity at 70 per cent. The only source of light in the room was artificial. Infected stocks of mosquitoes were kept in a screened cubicle equipped with an entrance having a double trap. Two types of cages for mosquitoes were designed for and used in this work. These have been described in a separate paper (Barnett, 1955).

Standard techniques were used unless otherwise stated. Concentrations of virus are expressed as the exponent of the reciprocal of the LD_{50} dilution as calculated by the method of Reed and Muench (1938).

RESULTS

General. During this study, 131 transmissions of Western equine encephalitis virus were obtained. Of this number, 124 were obtained from 252 routine attempts at transmission, and 7 during the course of 78 attempts at transmission designed to obtain special information. From the entire series of 131 transmissions, 29 (22 per cent) of the agents transmitted were re-identified by neutralization tests. These re-identifications included 26 agents transmitted in the routine series, two in an interrupted blood meal series, and one in a mechanical transmission. Eighty transmissions obtained in this study were from canary to chicken, 49 from chicken to chicken, and two were from canary to canary.

Of the 124 transmissions in routine attempts, 101 followed the bite of a single mosquito, while the remaining transmissions followed the bites of 2–14 mosquitoes. Three transmissions of an interrupted blood-meal series were each produced by the bite of a single mosquito, as were three transmissions in an experiment on the minimal infecting dose of virus. The single mechanical transmission followed the bites of two mosquitoes.

The minimal amount of virus necessary to infect mosquitoes. One experiment was performed to determine the minimal amount of virus necessary to infect mosquitoes. Seven 25-day-old chicks were inoculated subcutaneously with virus and each was then bled for titration at two-hour intervals ranging from seven to fifteen hours after the inoculations. Immediately after blood was drawn, each chick was placed in a cage with a group of normal mosquitoes, where they remained for periods up to one-half hour. The seven lots of mosquitoes were subsequently offered blood-meals on normal chicks 9, 10, 20 and 31 days after the mosquitoes had received the infective blood-meal. Unfortunately, the mosquitoes showed little tendency to refeed, and consequently, the data are scanty. The results are given in Table 1, where it will be noted that mosquitoes which had

TABLE 1

Results of an experiment on the minimal amount of virus necessary to infect Culex tarsalis

LD_{50} virus titer of infecting chick	Number of attempted transmissions	Number of transmissions
Less than 1.0	6	0
1.8	7	0
2.5	1	0
2.8	1	0
3.2	1	1
4.2	2	1
more than 6.0	2	1
Totals	19	3

fed on a chick with an LD_{50} virus titer of 3.2 subsequently transmitted virus, whereas those which had fed on chicks with lower titers failed to transmit virus. Since the number of transmission attempts in this experiment was very small the determination of the minimal amount of virus necessary to infect *C. tarsalis* is probably only an approximation.

Three transmissions were obtained from mosquitoes which had taken only partial blood meals from the chick serving as the virus donor. One of these mosquitoes had taken only a trace of blood from a chick which had an LD_{50} virus titer of 5.6. Two of the transmissions occurred seven days after and one occurred 20 days after the infective blood-meal.

The number of mosquito bites necessary to infect birds. Of the entire series of 131 transmissions, 107 followed the bite of a single mosquito. It is, therefore, amply evident that the bite of a single infective mosquito is sufficient to produce viremia in birds. In one instance, transmission of virus occurred after a single infective mosquito merely drew a trace of blood from a normal chick.

On four occasions, the LD_{50} virus titers of birds which had been infected by the bite of a single mosquito were determined. LD_{50} virus titers of two chicks obtained by bleeding the chicks approximately 24 hours after they were bitten by mosquitoes, were 5.4 and 5.6, and the LD_{50} virus titers in two canaries infected and bled in the same manner were 6.4 and 8.5. It should be noted that the last titer is as high as the highest produced in a canary in this study by subcutaneous inoculations of a 10^{-5} dilution of stock mouse brain virus.

The extrinsic incubation period and the duration of infectivity. The minimal extrinsic incubation period obtained in this study, with mosquitoes maintained at 24°C. (75°F.), was four days. All mosquitoes which had received infected blood meals subsequently were offered normal chicks daily, but no blood-meal was taken until the third day. On the third day after the infective blood meal, 26 individual mosquitoes fed on normal chicks but all failed to transmit virus. However, 10 per cent of all mosquitoes refeeding on the fourth day after the infective blood meal succeeded in transmitting virus. It is of interest to note that the minimal extrinsic incubation period obtained in this study coincided closely with the interval between blood-meals taken by *C. tarsalis* under laboratory

TABLE 2

Distribution of the extrinsic incubation periods of fifty-seven Culex tarsalis *infected with Western equine encephalitis virus*

Number of days after infective blood meal	Frequency of occurrence	Per cent of total
3	0	0
4	4	7
5	4	7
6	11	19
7	3	5
8	4	7
9	15	26
10	6	11
11	5	9
12	3	5
13	2	4
14	0	0
Totals.....	57	100

Mean—8.2 days; Median—9 days; Mode—9 days.

conditions. The mean interval between blood-meals was found to be 4.8 days, while the median and mode were both 4 days.

The distribution of extrinsic incubation periods in 57 individual mosquitoes infected with Western equine encephalitis virus is presented in Table 2. The mean incubation period was 8.2 days, and both the median and mode were 9 days.

Transmissions were obtained as late as 60 days after the infective blood-meal. This is as late as it was found possible to obtain refeeds from surviving infective mosquitoes, and from these results it is presumed that mosquitoes can remain infective for life.

The relation of the time interval after the infective blood-meal to the rate of transmission. In a series of 220 transmission attempts with single infected mosquitoes, the rate of transmission showed a gradual increase, beginning at 10 per cent on the fourth day after the infective blood-meal, and extending to the twelfth day, after which the rate of transmission stabilized at about 84 per cent. This is shown in Table 3. No correlation could be found between the age of mosquitoes at the time of the infective blood-meal and their subsequent rates of infection.

The frequency of transmission by individual mosquitoes. The frequency of transmission is limited, quite naturally, by the frequency with which a mosquito will refeed. Some mosquitoes refused to refeed after the initial blood-meal, but one re-fed as often as five times in a 14-day period, and another mosquito re-fed six times in a 16-day period.

The transmission histories of sixteen mosquitoes which had re-fed three or more times have been compiled and are presented in Table 4. From this table it can be seen that individual mosquitoes will frequently transmit as often as they

TABLE 3

Relation of time after infective blood-meal to the rate of transmission of virus by single Culex tarsalis

Days after infective blood meal	Number of attempted transmissions	Number of transmissions	Per cent positive
3	26	0	0
4	42	4	10
5	14	4	29
6	27	12	44
7	6	2	33
8	12	8	66
9	27	18	66
10	12	9	75
11	9	7	77
12	7	5	71
13–60	38	32	84
Totals.....	220	101	46

refeed, once the extrinsic incubation period has been passed. It should also be noted that on three occasions, mosquitoes which had transmitted virus (Nos. 38-2R, 34-4R, and 50-4R) failed to do so in subsequent feedings. The explanation for these failures is not apparent. No evidence was obtained during this study to indicate that any of the chicks were refractory to infection with Western equine encephalitis virus, and nothing is known concerning fluctuations in the

TABLE 4

Frequency of virus transmission by sixteen Culex tarsalis *which re-fed three or more times*

Designation of mosquito	Number of days after infective blood-meal when refeeding occurred, with transmission results*
♀19-1R	**(6)** **(11)** **(14)**
♀5-2R	*4* *8* **(11)**
♀38-2R	**(5)** **(8)** *11* **(13)**
♀2-3R	*6* 9 *12*
♀14-3R	*3* 9 **(13)**
♀17-3R	*3* *6* 9
♀25-3R	*4* *8* *11*
♀7-4R	*4* **(9)** **(14)**
♀12-4R	*3* **(9)** **(12)** **(14)**
♀13-4R	*3* **(6)** **(10)** **(13)** **(16)**
♀15-4R	*3* **(10)** **(14)**
♀16-4R	*3* **(9)** **(14)**
♀34-4R	*3* **(9)** *12*
♀45-4R	**(5)** **(9)** **(14)** **(17)**
♀49-4R	*3* **(7)** **(9)** **(13)**
♀50-4R	*3* **(10)** *14*

* Numbers in bold type represent transmissions, italicized numbers represent failures to transmit.

virus titer of a given mosquito during its lifetime. It is possible that something in the feeding process of these mosquitoes was responsible for these failures to transmit, but, as was indicated earlier in this paper, only a partial blood-meal by a mosquito can result in the transmission of virus.

Transovarian passage of virus. Attempts to recover virus from the eggs and progeny of laboratory infected *C. tarsalis* females by Hammon and Reeves (1945) were uniformly unsuccessful. Hammon and Reeves (1947) also failed to recover virus from 2,485 wild-caught male *C. tarsalis* and from 131 females which had been reared from larvae. They interpreted the field data as possible evidence negating the likelihood of transovarian passage of virus.

In our work, seventeen attempts were made to determine whether or not the progeny of five lots of infective mosquitoes could transmit virus to chicks through their bites. Progeny of infective mosquitoes were tested from two days after emergence from the pupal stage to as late as 26 days after emergence. The number of mosquitoes biting chicks in these tests varied from 1 to 18. All attempts to demonstrate transovarian passage were unsuccessful.

Mechanical transmission of virus. Three experiments were conducted to determine if *C. tarsalis* could transmit virus from bird to bird mechanically. In the first experiment, a 10-day-old chick was inoculated with virus and 24 hours later a blood sample was drawn for virus titration, the result of which was an LD_{50} virus titer of 5.6. Immediately after the bleeding for titration, the infected chick was placed in a cage of normal mosquitoes. As soon as mosquitoes were observed feeding, and in some instances merely probing, they were removed by aspirator and deposited in a second cage containing a normal 11-day-old chick. In this manner, a large number of mosquitoes were interrupted while feeding and distributed in six cages, each of which contained a normal chick. All six normal chicks were subsequently either fed upon or probed by one or more mosquitoes. The six chicks were then held for 24 hours when they were bled, and a 10^{-1} dilution of whole blood from each was then inoculated intraperitoneally into a litter of 4-day-old suckling mice. Five of the six birds failed to show evidence of viremia, but the blood from the sixth bird killed five mice on the second day after inoculation, while two remaining mice showed distinct central nervous system signs. The two sick mice were sacrificed and a 10^{-1} suspension of mouse brain was prepared. Western equine encephalitis virus was demonstrated in this suspension by neutralization test. Only two mosquitoes had attempted to feed on the bird from which virus was recovered. The first had merely probed the infected chick, no sign of ingested blood being visible, and then probed the normal bird for five minutes. The second mosquito which had partly engorged on the infected chick probed the normal bird for three minutes. Neither of these mosquitoes appeared to draw any blood from the second chick, and the interval between bites on the infected bird and on the normal bird was less than twenty minutes.

In the second experiment, a 29-day-old chick with an LD_{50} virus titer of 5.6 was used as the virus donor and thirteen 15-day-old chicks were used as normal recipient birds. From one to six mosquitoes which had been interrupted in feeding

on or in probing the infected chick subsequently fed upon or probed each of the thirteen normal birds. No transmission of virus occurred.

In a third experiment, an infected canary with an LD_{50} virus titer of 7.5 was used as the virus donor. One or two mosquitoes which had been interrupted in feeding on or in probing the infected canary subsequently fed upon or probed each of five normal canaries. One transmission may have occurred. In this instance, two of six suckling mice showed typical encephalitis signs on the second day and died on the third day following inoculation of a 10^{-1} dilution of blood from a recipient canary. A third mouse developed typical symptoms on the third day and died on the fourth day. No further mortalities occurred, and none of the remaining mice developed typical encephalitis symptoms. Unfortunately because of a miscalculation in time, no passage of brain tissue from the sick mouse had been made on the third day and, therefore, the only possible follow-up on this litter was a blind passage made from two mice on the fourth day. This blind passage failed to produce any mortality in a litter of seven 4-day-old mice.

These results suggest that mechanical transmission of virus may occur in nature, but there is no reason to believe that the ability to transmit virus in this manner is limited to *C. tarsalis* or to mosquitoes in general, since interrupted feeding occurs in many blood-sucking arthropods.

The effects of low temperature on the titer of virus in Culex tarsalis. The reservoir of Western equine encephalitis virus is unknown, but it is not an unreasonable hypothesis that the mosquito vector of this virus may also serve as the reservoir. Attempts to isolate virus from overwintering *C. tarsalis* in Kern County, California, and in the Yakima Valley, Washington, by Hammon *et al.* (1945, 1945a) and by Hammon and Reeves (1947) ended in failures. More recently, Reeves (communication) reported virus isolations from *C. tarsalis* which were collected in October and tested for virus after several months in captivity. However, this work was done in Kern County, California, where winters are relatively mild.

Observations by Keener (1952) in western Nebraska have indicated that *C. tarsalis* females could be collected in small numbers in food storage cellars throughout the winter months. The temperature within these cellars, where specimens were regularly found, did not go below 1°C. (33°F.) and averaged about 5°C. (41°F.) during the winter months. To facilitate studies on the persistence of virus in overwintering mosquitoes under natural conditions, a root cellar similar to those described by Keener (*op. cit.*) was constructed on the grounds of the Rocky Mountain Laboratory. This root cellar was built into the side of a hill, the interior finished in rough lumber, and the top and sides covered with two feet of earth. A hygrothermograph placed in the root cellar during the period of operation, which extended from November to February, indicated that both temperature and relative humidity in the root cellar were remarkably stable, despite outdoor weather conditions. The temperature remained at 6.5°C. (44°F.), while the relative humidity remained at 65 per cent.

This root cellar was then stocked at intervals with eleven lots of infected mosquitoes during the month of December. Nine of the lots had fed on infected chicks, two on canaries. Two of the lots were "winter-conditioned" by holding

the pupae in a refrigerator for two days prior to the emergence of adults, and a third lot was "winter-conditioned" by holding the adults for three days in a room where the temperature was intermediate between that of the insectary and that of the root cellar, prior to placing them in the root cellar. Lots varied in size from 5 to 365 mosquitoes. Nine lots were held in large cages of a variety of types and sizes, some of which also contained loose straw. One lot of five mosquitoes was held in a gauze-covered quart jar, and another lot was distributed individually in small cellulose acetate containers, some of which had been provided with water.

All of the lots suffered high mortalities during the first week in the root cellar and within two weeks 50 per cent of each lot had died. After one month, almost 100 per cent mortality had occurred, and the mosquitoes surviving longest lived only 43 days.

Following the failure of the long-term experiment on the ability of overwintering mosquitoes to retain and transmit virus at the end of a winter, an experiment was performed to determine the short-term effects of temperature on the titer of virus in *C. tarsalis*. The organization of this experiment, which was somewhat complicated, has been diagramed in Figure 1, together with the results. A large lot of 6-day-old normal *C. tarsalis* which had fed on a chick with an LD_{50} virus titer of 4.5 was divided into two sublots. Sublot # 1 was held in the insectary at 24°C. for seven days, after which two pools of ten mosquitoes each were triturated and titrated for virus. These yielded LD_{50} virus titers of 6.3 and greater than 7.0. This sublot was then further subdivided on the seventh day. Sublot # 1A was held in the insectary for seven additional days, and sublot # 1B was held in the root cellar at a temperature of 6.5°C. for seven days. Sublot # 2 was held in the root cellar at 6.5°C. for seven days, at the end of which two pools of ten mosquitoes each were taken and titrated for virus. These yielded LD_{50} virus titers

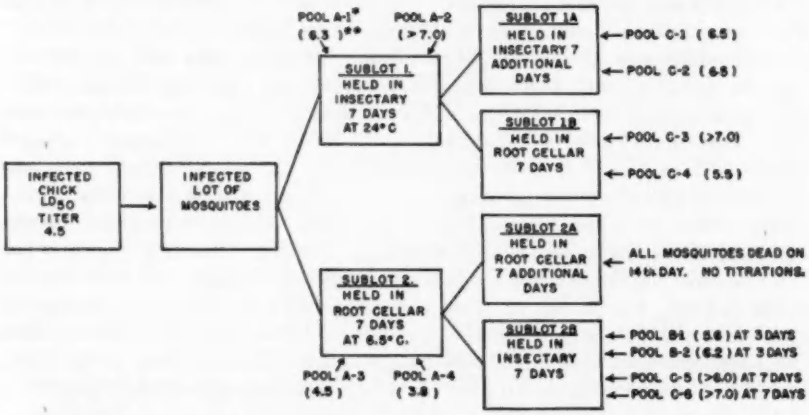
INFECTED CHICK LD50 TITER 4.5
INFECTED LOT OF MOSQUITOES
POOL A-1* (6.3)** POOL A-2 (>7.0)
SUBLOT 1. HELD IN INSECTARY 7 DAYS AT 24°C
SUBLOT 1A HELD IN INSECTARY 7 ADDITIONAL DAYS
← POOL C-1 (6.5)
← POOL C-2 (6.5)
SUBLOT 1B HELD IN ROOT CELLAR 7 DAYS
← POOL C-3 (>7.0)
← POOL C-4 (5.5)
SUBLOT 2. HELD IN ROOT CELLAR 7 DAYS AT 6.5°C.
SUBLOT 2A HELD IN ROOT CELLAR 7 ADDITIONAL DAYS
← ALL MOSQUITOES DEAD ON 14th DAY. NO TITRATIONS.
SUBLOT 2B HELD IN INSECTARY 7 DAYS
← POOL B-1 (5.6) AT 3 DAYS
← POOL B-2 (6.2) AT 3 DAYS
← POOL C-5 (>6.0) AT 7 DAYS
← POOL C-6 (>7.0) AT 7 DAYS
POOL A-3 (4.5) POOL A-4 (3.8)
* ALL POOLS CONSISTED OF 10 MOSQUITOES EACH
** FIGURES ENCLOSED IN PARENTHESES INDICATE THE LD50 TITERS OBTAINED FROM POOLS.

FIG. 1. The effect of temperature on the titer of WEE virus in *Culex tarsalis*.

of 4.5 and 3.8. Sublot #2 was then further subdivided on the seventh day into two components: Sublot #2A was held in the root cellar for seven additional days, and sublot #2B was held in the insectary for seven days.

After sublot #1A had been held in the insectary a total of fourteen days, two pools of ten mosquitoes each yielded LD_{50} virus titers of 6.5. After sublot #1B had been held in the insectary for seven days and then in the root cellar for seven days, two pools of ten mosquitoes each, titrated for virus at the end of this period, yielded LD_{50} virus titers of 5.5 and greater than 7.0.

All mosquitoes in sublot #2A were found dead on the fourteenth day in the root cellar, and no virus titrations on this sublot were made. Two pools of ten mosquitoes each were taken from sublot #2B after this sublot had been in the insectary three days following a seven-day holding period in the root cellar. These two pools yielded LD_{50} virus titers of 5.6 and 6.2. After four additional days in the insectary, two more pools of ten mosquitoes each were taken from sublot #2B and titrated for virus. These pools yielded LD_{50} virus titers of greater than 6.0 and greater than 7.0.

The results indicate that virus multiplication occurs in mosquitoes, because several of the mosquito pools yielded virus titers greater than the titer of the virus donor chick. The results also show that virus titers will be higher in moquitoes held at high temperatures than in mosquitoes held at low temperatures. Lastly, the evidence fails to show that virus titers in mosquitoes drop when infected mosquitoes which had been held at high temperatures are suddenly removed to an environment with a low, sub-optimal temperature, at least when they are held at such sub-optimal temperatures for periods up to one week.

The retention of virus by dead Culex tarsalis. In the attempted recovery of virus from wild-caught mosquitoes, the question whether or not to include dead mosquitoes in the mosquito pools being tested frequently arises. Light-trap catches often contain large numbers of dead mosquitoes, and sometimes large proportions of material collected in the field by other methods are dead by the time of arrival at the laboratory. Separation of the dead material from living material can be very laborious. The availability of large numbers of *C. tarsalis* and the practical importance of this question prompted a brief study of the retention of virus in dead mosquitoes.

A large lot of normal *C. tarsalis* was permitted to feed on a chick with an LD_{50} virus titer of 5.6. Six days after the infective blood-meal, a large number of these mosquitoes were killed with chloroform and thereafter held in a room where the temperature ranged from 22–24°C. and the relative humidity varied from 47–56 per cent. Immediately, after death, two pools of ten mosquitoes each were triturated and titrated for virus. These pools yielded LD_{50} virus titers of 5.6 and 6.4. Four hours later, another pool of ten dead mosquitoes was titrated for virus and this yielded an LD_{50} virus titer of 4.8. A pool of ten dead mosquitoes titrated eight hours after death produced an LD_{50} virus titer in excess of 5.0 (at least 5.5) and a fifth and final pool of ten dead mosquitoes, titrated 24 hours after death, gave an LD_{50} virus titer greater than 5.0 and at least 5.5. It should be noted that in these tests, all mosquitoes presumably were infected. From the data available,

it appears that virus persists in dead mosquitoes at high titers for periods up to 24 hours after death, and that it is not necessary to separate recently dead mosquitoes from living ones when attempting to isolate Western equine encephalitis virus from wild-caught *C. tarsalis*.

DISCUSSION

This study has confirmed and extended the findings of Hammon and Reeves (1943, 1946) on the transmission of Western equine encephalitis by *Culex tarsalis*. In the Hammon and Reeves investigations, the shortest extrinsic incubation period demonstrated was ten days and *C. tarsalis* was shown to remain infective for thirty days, which was the length of their longest experiment. By contrast, this investigation, which was based on a series of 131 transmissions, demonstrated a minimal incubation period of four days. It was also found that *C. tarsalis* could remain infective for as long as sixty days, suggesting that this mosquito can remain infective for life.

Several findings of this investigation demonstrate the effectiveness of *C. tarsalis* as a vector of Western equine encephalitis under laboratory conditions. This is illustrated by the finding that 84 per cent of *C. tarsalis* transmitted virus thirteen days after an infective blood-meal, and by the frequency with which a bite from a single infective mosquito produced viremia in birds. The effectiveness of this mosquito as a vector under laboratory conditions was enhanced by a short extrinsic incubation period, which can be as brief as four days. The finding that the minimal extrinsic incubation period coincides closely with the average interval between blood meals, suggests a further biological factor favoring virus transmission. Other findings which tend to support the importance of this species as a vector have been the observation that most mosquitoes transmit virus as often as they refeed, once the extrinsic incubation period has been passed, and the data suggesting that once a mosquito becomes infective it remains so for life.

It is believed that this study demonstrated, for the first time, mechanical transmission of Western equine encephalitis virus by an arthropod. The importance of this phenomenon in nature is not known, but the infrequency with which mechanical transmission was obtained in this study suggests that it plays a minor role in the dissemination of the virus.

The failure to maintain *C. tarsalis* females through the winter season in a root cellar should not be interpreted as evidence that infected mosquitoes cannot survive under the conditions provided. Virtually nothing is known concerning the natural processes which condition mosquitoes for hibernation, or if such natural conditioning even occurs. Similarly, nothing is known concerning the survival rate of those mosquitoes in nature which do enter suitable hibernation quarters. Study of the factors, if any, predisposing to mosquito hibernation in nature should properly precede experiments of the type here described.

SUMMARY

This investigation confirmed and extended the work of Hammon and Reeves (1943) which demonstrated that the mosquito *Culex tarsalis* is capable of trans-

mitting Western equine encephalitis virus from bird to bird. Virus was transmitted from chicken to chicken, from canary to canary, and from canary to chicken; 131 transmissions of virus were obtained in the study, and 29 (22 per cent) of the agents transmitted were re-identified by neutralization test.

Infectivity was acquired by these mosquitoes only when they fed on a bird with a threshold, or greater, level of viremia. This threshold level, of viremia was shown to be not more than an LD_{50} titer of 3.2. The minimal extrinsic incubation period was found to be four days, and it was further demonstrated that the rate of transmission steadily increased from 10 per cent on the fourth day to 84 per cent after the thirteenth day following the infective blood-meal. Transmission was readily accomplished by the bite of a single infective mosquito, and virus was transmitted by such a mosquito as often as four times in eleven days. Virus transmission was obtained from mosquitoes as late as sixty days after they had had an infective blood-meal, and from these results it is presumed that mosquitoes can remain infective for life.

Negative results were obtained in all attempts to demonstrate transovarian passage of western equine encephalitis virus in *C. tarsalis*. One mechanical transmission of virus by *C. tarsalis* was obtained in 24 attempts, suggesting that this phenomenon may also occur in nature, but the epidemiologic significance is not known.

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EXPERIMENTAL STUDIES OF CONCURRENT INFECTION OF CANARIES AND OF THE MOSQUITO *CULEX TARSALIS* WITH *PLASMODIUM RELICTUM* AND WESTERN EQUINE ENCEPHALITIS VIRUS[1, 2]

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The mosquito *Culex tarsalis* plays an important role in the dissemination of Western equine encephalitis virus (Hammon *et al.*, 1941, 1945, 1945a) and has also been shown to be a very efficient vector of the avian malaria parasite *Plasmodium relictum* (Huff, 1927, 1932; Ball, 1947; Rosen and Reeves, 1954). Further, recent studies by Reeves *et al.* (1954) have indicated that this mosquito is one of the species most commonly infected with avian malaria in Kern County, California, an area in which epidemics and epizootics of Western equine encephalitis have frequently occurred. This association of viral and protozoan agents in a common vector has logically led to the question, "what effect, if any, does infection of *C. tarsalis* with avian malaria plasmodia have on its ability to transmit Western equine encephalitis virus?"

A six-year study by Herman *et al.* (1954) in Kern County, California indicated that avian malaria is very common in the wild bird population, and that *Plasmodium relictum* is the commonest malaria parasite of birds. Since there is strong evidence indicating that birds are the primary hosts of Western equine encephalitis virus, and since little is known concerning the reservoir of the virus, speculation has arisen that plasmodia in birds suffering concurrent infections with the virus and avian malaria might acquire the virus, retain it over a considerable period of time, and thus, possibly serve as the virus reservoir. Malaria relapse in such birds might conceivably serve as the mechanism re-establishing the virus in nature from epidemic season to season. This study was undertaken to examine these possibilities through laboratory investigation.

MATERIALS AND TECHNIQUES

The strain of malaria used in this work is the *1P1-1* strain of *Plasmodium relictum*. The history and characteristics of the virus strain used have been given in a previous paper (Barnett, 1955) together with details of the inoculation of

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laboratory animals with virus, methods used in the transmission of virus by mosquitoes, the history of the mosquito strain used, and the conditions under which mosquitoes were maintained. Canaries were used almost exclusively in this study because they are highly susceptible to infection with both the virus and the plasmodium, yet do not develop clinical illness which might interfere with long-term observation. They were infected with virus by subcutaneous inoculation of 0.05 cc. of a 10^{-5} dilution of stock virus, and were usually infected with plasmodia by intramuscular inoculation of approximately 0.05 cc. of heparinized infected canary blood. Occasional transmission of the plasmodia strain was made from canary to canary through *C. tarsalis*.

Concentrations of virus are expressed throughout this paper as the exponent of the reciprocal of the LD_{50} dilution as calculated by the method of Reed and Muench (1938). Three to four day-old suckling white mice were used exclusively for the determination of virus titers.

COMPARISON OF THE TRANSMISSION OF WESTERN EQUINE ENCEPHALITIS VIRUS BY *CULEX TARSALIS* (a) CONCURRENTLY INFECTED WITH *PLASMODIUM RELICTUM* AND THE VIRUS, AND (b) INFECTED WITH THE VIRUS ONLY

Two lots of *C. tarsalis* females infected concurrently with avian malaria and virus were compared with two lots of mosquitoes infected with virus only. The infection histories of these lots were as follows: Lot #1, which consisted of 70 mosquitoes, fed on a canary with a parasite count of 3,116 per 10,000 red blood cells and an LD_{50} virus titer of 3.8; lot #2, which consisted of 80 mosquitoes, fed on a canary with a parasite count of 1,456 per 10,000 red blood cells and an LD_{50} virus titer of 5.6; lot #3, which consisted of 25 mosquitoes, fed on a malaria-free canary with an LD_{50} virus titer of 6.2; and lot #4, which consisted of 50 mosquitoes, fed on a malaria-free canary with an LD_{50} virus titer of 7.5. All subsequent work with these four lots of mosquitoes was done using individual mosquitoes.

To check on the proportion of mosquitoes in lots #1 and #2, which actually were infected with avian malaria, twenty mosquitoes from lot #1 and thirty mosquitoes from lot #2 were dissected seven and eight days respectively after the infective blood-meal. All mosquitoes in lot #1 showed oocysts on the gut and all but one of the mosquitoes in lot #2 showed oocyst production.

A more uniform distribution of LD_{50} virus titers in the canaries used could not be obtained because, as will be shown later, concurrent infection of canaries with plasmodia and virus suppresses the level of viremia in these canaries. This introduced a second variable into the experiment. Subsequently a third uncontrollable variable developed when the four lots of mosquitoes showed variation in their tendencies to feed on normal birds. This variation resulted in a skewed distribution of attempted transmissions among the four lots of mosquitoes with respect to the time of feeding after the infective blood-meal.

The results obtained in attempted transmissions with lots #1 and #2 have been combined and compared with the combined results from lots #3 and #4. These results, presented in Table 1, show the daily variation in the percentage of

TABLE 1

Comparison of the transmission of western equine encephalitis virus by Culex tarsalis *concurrently infected with* Plasmodium relictum *and the virus, with* Culex tarsalis *infected with the virus only*

No. days after virus infection of mosquitoes	Transmission attempts by mosquitoes infected with malaria and virus			Transmission attempts by mosquitoes infected with virus only		
	Number of attempts	Number positive	Per cent positive	Number of attempts	Number positive	Per cent positive
3	18	0	0	5	0	0
4	22	0	0	13	3	23
5	2	1	50	8	3	38
6	4	2	50	20	9	45
7	2	1	50	1	0	0
8	3	1	33	7	5	71
9	25	17	70	2	1	50
10	9	6	67	1	1	100
11	3	2	67	6	5	83
12	5	3	60	1	1	100
13	4	4	100	1	1	100
14	9	8	89	1	1	100
Totals.....	106	45	42	66	30	45

transmissions by mosquitoes infected with virus only and by those infected with both agents. It will be noted that mosquitoes infected with both agents transmitted in 42 per cent of all attempts while those infected only with virus had a comparable transmission rate of 45 per cent. However, when the results from the four lots are examined separately, a discrepancy becomes apparent. Lot #1 transmitted in only 13 per cent of all attempts, while lot #2 transmitted in 65 per cent of all attempts. Lots #3 and #4 were comparable, with rates of transmission of 46 per cent and 45 per cent, respectively. The difference in rates of transmission shown by lots #1 and #2 could be attributed to differences in virus titer in the birds upon which the mosquitoes fed since lot #1 fed on a bird with a titer of only 3.8 whereas lot #2 fed on a bird with a titer of 5.6. This explanation does not apply to the discrepancies between the transmission rate of lot #2 and the transmission rates of lots #3 and #4, since all three lots had fed on birds with comparable virus titers. The probable explanation lies in an examination of the raw data which shows that 65 per cent of all attempted transmissions by lot #2 occurred eight or more days after the infective blood-meal whereas in lots #3 and #4, only 28 per cent and 29 per cent of the attempted transmissions occurred eight or more days after the infective blood-meal, respectively. It has been pointed out in a previous publication (Barnett, 1955), that the rate of transmission varies directly with the length of the interval between infective blood-meal and attempted transmission. Therefore, it is apparent that the data obtained from lot #2 are not strictly comparable with the data obtained from lots #3 and #4. The much higher proportion of late attempted transmissions obtained from lot #2 suggests that lot #2 should have

had a higher rate of transmission than either lots #3 and #4, and this, apparently, has occurred.

From these results, it appears that the variation in the rates of transmission of the four lots of mosquitoes was related to virus titer in the infecting canaries and to time of feeding by mosquitoes rather than to the presence or absence of concurrent plasmodial infection in the mosquitoes. It should also be noted, as shown in Table 1, that only minor variation occurred in the minimal extrinsic incubation periods of the two types of infected mosquitoes. Similarly, transovarial transmission of virus could not be demonstrated in either mosquitoes infected with virus only, or in mosquitoes infected with both agents.

STUDIES ON CONCURRENT INFECTION OF CANARIES WITH *PLASMODIUM RELICTUM* AND WESTERN EQUINE ENCEPHALITIS VIRUS

The effects of malarious canary blood on suckling white mice. Before undertaking studies on the blood of canaries concurrently infected with plasmodia and Western equine encephalitis virus, it was desirable to learn whether the blood of malaria-infected canaries was detrimental to suckling white mice. Accordingly, tests were made on three litters of suckling mice with blood from two canaries with patent infections of *P. relictum*. Each of a litter of six 7-day-old mice was inoculated intracerebrally with 0.03 cc. of a 10^{-1} dilution of malarious canary blood in 10 per cent rabbit serum-saline. This blood contained 560 parasites per 10,000 red blood cells. One mouse died within five minutes after the inoculation and a second mouse died four days after the inoculation; all others survived for seven days. The same dilution of blood was also used to inoculate a litter of seven 4-day-old suckling mice intraperitoneally, each mouse receiving 0.05 cc. of inoculum. All seven mice survived for seven days. Each of a litter of six 5-day-old suckling mice was then inoculated intraperitoneally with 0.05 cc. of a 10^{-1} dilution of malarious blood containing 1,202 parasites per 10,000 red blood cells, in a third test. One mouse died on the third day following inoculation, and all others survived seven days. These results indicated that little or no mortality could be expected in suckling mice inoculated with the blood of canaries suffering patent malaria infections.

The distribution of virus in the blood components of infected chicks. As a preliminary to a study of the distribution of virus in the blood components of canaries, some information was desired on the dist ibution of virus in the blood of chicks to serve as base-line data. Accordingly, two 18-day-old chicks were inoculated subcutaneously with 0.05 cc. of a 10^{-5} dilution of stock virus, bled 21 hours later, the blood was separated into plasma and cells, the cells washed four times and the cells and plasma titrated for virus. The blood cells yielded an LD_{50} virus titer of less than 1.0 and the plasma produced an LD_{50} virus titer of 5.2.

The distribution of virus in the blood components of canaries. From the results obtained with chicks it appeared that an increase in the virus titer of the blood cells of birds following the possible acquisition of virus by plasmodia should be reflected in titrations of red blood cells performed in suckling mice. Therefore, an experiment was undertaken in which pooled blood from three canaries, which

had been inoculated with virus at or near peak malaria parasitemia was separated into its component plasma and blood cells and then each component was titrated after the cells had been washed five times. Similar titrations were performed at the same time on the plasma and blood cells from pooled blood samples drawn from three canaries infected with virus only. Blood samples were drawn from both groups of birds 24 hours after the virus inoculation. Parasite counts, at the time of blood withdrawal, on smears from each of the three malaria and virus infected canaries were 326, 375, and 731 per 10,000 red blood cells. The LD_{50} virus titer of the blood cells from canaries concurrently infected with plasmodia and virus was 3.5 while the plasma from these birds yielded an LD_{50} virus titer of 6.6. Blood cells from the second pool of blood (birds infected with virus only) yielded an LD_{50} virus titer of 1.9 while an LD_{50} virus titer of 7.4 was obtained from the plasma. Thus, it appeared that, although the plasma virus titer of canaries concurrently infected with plasmodia and virus was lower than the plasma virus titer of canaries infected solely with virus, the virus titer of the blood cells in the former group of birds was significantly higher than that of the canaries infected with virus only.

This experiment was conducted a second time using four canaries in each group. In all other respects the experiment was performed in a similar manner. Parasite counts, at the time of blood withdrawal, in the four birds concurrently infected with virus and plasmodia were 247, 318, 1,016, and 1,275 per 10,000 red blood cells. The blood cells of the canaries concurrently infected with malaria and virus produced an LD_{50} virus titer of 1.2, and the plasma of these birds yielded an LD_{50} virus titer of 4.3. On the other hand, the blood cells and plasma of the canaries infected solely with virus yielded LD_{50} virus titers of 2.8 and 7.3 respectively. Thus, the results conflicted with those obtained in the previous experiment, and since Hammon (communication) pointed out at this time that he had obtained grossly irregular results in previous unpublished work in which he had performed replicate titrations on the blood components of chicks and guinea pigs infected with Western equine encephalitis virus, it was decided to drop this approach to the problem.

The duration of viremia in canaries. To determine whether or not virus was retained by plasmodia for any length of time, the duration of viremia in six canaries which had been inoculated with virus at or near peak malaria parasitemia was determined. The duration of viremia in five control canaries infected with virus only was also determined.

Five of the six canaries with concurrent infection had virus circulating in the blood stream for five days after the virus inoculation. None of these five birds gave any evidence of circulating virus on the sixth day despite the fact that parasites still persisted in the blood of four of them six or more days after virus inoculation. The duration of viremia in the sixth bird in this group was only four days, despite the persistence of parasites in the red blood cells.

In the five control birds, viremia lasted three to four days, but two of the birds were not tested until the fifth day and no information was obtained from these birds since they were negative when first tested.

These results fail to show that Western equine encephalitis virus can persist for more than five days in canaries infected with *Plasmodium relictum*, nor can it be inferred that virus is associated with the parasites during this time.

Attempts at inducing artificial malaria relapses in canaries. Early in this study it was planned to induce artificial malaria relapses in canaries which had been concurrently infected with avian malaria and virus, and which were in the latent stage of malaria infection. Whitmore (1918) reported that the injection of foreign proteins would produce relapses, and Ben-Harel (1923) reported successful results following the injection of adrenalin.

Attempts were first made on five birds in the latent stage of malaria (no previous infection with encephalitis virus). These birds were inoculated subcutaneously with adrenalin hydrochloride diluted in isotonic saline in doses varying from 0.05 cc. of a 10^{-5} dilution to 0.1 cc. of a 10^{-3} dilution. None of these treatments induced a malaria relapse.

Four canaries in the latent stage of malaria infection (no previous infection with encephalitis virus) were then inoculated with various doses of undiluted bovine serum. The first of these birds received 0.02 cc. subcutaneously, the second received 0.30 cc. subcutaneously, and the third and fourth each received 0.05 cc. intravenously. Only the second bird subsequently showed evidence of a relapse. One gametocyte was observed in a blood smear prepared from this bird eight days after the bovine serum inoculation, but parasites were not observed in blood smears taken prior to or after the eighth day.

Following the failures to induce artificial relapses in birds with latent malaria infections, eight remaining canaries which had recovered from concurrent viremia and patent malaria infection were sacrificed and the spleens removed. The spleens of these birds were triturated individually and 10^{-1} suspensions of each were inoculated into litters of four-day-old suckling mice. Virus was not isolated from any of the spleens, thus failing to demonstrate that plasmodial forms found in the spleen during malaria latency were harboring Western equine encephalitis virus.

Viremia levels attained in canaries concurrently infected with avian malaria and Western equine encephalitis virus and in canaries infected solely with the virus. Data on the level of viremia were obtained from twenty canaries infected with virus during the course of patent malaria infections and from twenty-one canaries infected with virus only. Blood was drawn for titration from each of these birds at approximately 24 hours after virus inoculation. Of the 21 birds infected with virus only, one titration was performed on the pooled blood of three birds, a second titration was performed on the pooled blood of four birds, and fourteen titrations were performed on individual blood samples from the remaining fourteen canaries. Similarly, of the twenty canaries concurrently infected with avian malaria and virus, one titration was performed on the pooled blood of three birds, a second titration was performed on the pooled blood of four birds, and thirteen titrations were performed on individual blood samples from the remaining thirteen canaries.

The two groups of canaries were similar in all respects except for the presence

or absence of concurrent patent infection with avian malaria. Canaries in both groups were taken at random, given the same dosage of stock virus by the same route of inoculation. Of the 41 canaries, 28 were tested in paired runs, i.e., when one or more canaries suffering malaria infection were inoculated with virus, a like number of normal canaries was inoculated at the same time and in the same manner. Similarly, birds in paired runs were bled for titration at the same time.

The LD_{50} virus titers obtained from the two types of infected birds are presented in Table 2. In calculating LD_{50} virus titers where an end point in the titration was not reached, it was assumed that no mortality would have occurred in the next higher dilution. Similarly, in titrations where no mortalities occurred in the lowest dilution, it was assumed that all mice in the next lower dilution would have died. An examination of the two columns of titer values indicates that the titer levels of birds concurrently infected with plasmodia and virus were almost uniformly lower than the titer levels obtained in birds with virus infection only, and that the respective mean values of the LD_{50} virus titers were 4.7 and 7.3.

The significance of this difference can readily be seen from the distribution of LD_{50} virus titers. The median LD_{50} virus titer of the entire series of titrations is 6.6. All fifteen LD_{50} virus titers in the first column of Table 2 fell below the median titer except two which are equal to the median titer. On the other hand,

TABLE 2

List of LD_{50} virus titers obtained from canaries concurrently infected with Plasmodium relictum *and Western equine encephalitis virus and from canaries infected with virus only*

LD_{50} virus titers in concurrent infections	LD_{50} virus titers in infections with virus only
1.5*	6.5
2.5*	6.6
2.5*	6.8
3.4	6.9
3.8	7.2
4.3‡	7.2
4.6	7.3‡
4.9	7.4
5.2	7.4†
5.6	7.5
6.2	7.5
6.4	7.5
6.5	7.5
6.6	7.5*
6.6†	7.5*
	8.5*
Mean = 4.7	Mean = 7.3

* Estimated titer

† Pool of blood from 3 birds

‡ Pool of blood from 4 birds

all LD_{50} virus titers in column 2 of Table 1, save two lie above the median titer, one of the remaining two titers being equal to the median titer, and the other being slightly below the median titer. These differences are so gross that it must be concluded that concurrent infection of canaries with plasmodia and Western equine encephalitis virus results in a suppression of virus titer 24 hours after inoculation in such canaries. Similar differences in virus titer were obtained when titrations were performed on blood samples drawn 48 hours after virus inoculation from six canaries concurrently infected with virus and plasmodia and from three canaries infected with virus only.

An examination of the characteristics of malaria infection and of LD_{50} virus titers in thirteen individual canaries inoculated with virus during the course of parasitemia, failed to show any distinct relationship between the level of parasitemia, either on the day of inoculation or on the day of titration, and the level of viremia attained. Similarly, no relation was found between the length of the patent period of malaria at the time of virus inoculation and virus titers attained. These data are presented in Table 3.

Evidence was obtained to indicate that no suppression of viremia occurs in canaries in the latent stage of malaria infection when such canaries are inoculated with virus. Four canaries which had been in the latent stage of malaria infection for periods of time ranging from six weeks to six months were inoculated with virus. All four birds subsequently developed high grade viremias with LD_{50} virus titers ranging from 6.8 to 7.8. It would appear, therefore, that the mechanism or entity responsible for the suppression of virus titers in birds suffering

TABLE 3

Relation of the degree of parasitemia and the length of the patent period of infection with Plasmodium relictum *at the time of inoculation of western equine encephalitis virus to the level of viremia attained in canaries*

Bird No.	Length of patent pd. of malaria infection at time of virus inocul. (in days)	Level of parasitemia*		LD_{50} virus titer
		At time of virus inocul.	At time of titration	
1	4	1373	160	1.5†
2	4	851	789	2.5†
3	5	2101	771	2.5†
4	2	218	531	3.4
5	3	1203	3116	3.8
6	4	2681	1421	4.6
7	3	1222	3021	4.9
8	4	1573	1951	5.2
9	2	824	1456	5.6
10	5	465	278	6.2
11	5	1020	482	6.4
12	4	3698	1446	6.5
13	6	1179	881	6.6

* Expressed as the number of parasites per 10,000 red blood cells

† Estimated titer

concurrent patent malaria infection and virus infection does not persist for periods of six weeks or more. Huff (1939) has reported that canaries infected with *Plasmodium relictum* or *P. cathemerium* do not develop higher body temperatures than do normal birds. Similarly, Sergent and Sergent (1946) found no difference between the body temperatures of healthy canaries and canaries infected with *P. relictum*. Therefore, it would appear that the suppression of viremia is not a result of pyrogenic action in the host.

Similar evidence was obtained to indicate that recovery from viremia does not prevent or suppress subsequent infection of canaries with *P. relictum*. Two canaries which had been inoculated with virus 39 days previously, and one which had received a virus inoculation 27 days previously were inoculated with 0.05 cc. of malarious blood. All three subsequently developed parasitemia with peak infection counts varying from 296 to 2,360 parasites per 10,000 red blood cells.

DISCUSSION

The data obtained in this study fail to support a hypothesis that avian malaria parasites acquire and retain Western equine encephalitis virus. The results obtained from virus titration of canary blood components were erratic and failed to establish that plasmodia infected blood cells yielded greater concentrations of virus than did blood cells from canaries infected with the virus only.

The findings that viremia does not continue beyond the fifth day after virus inoculation in canaries suffering concurrent infection with avian malaria and virus despite the persistence of parasitemia, argues against the stated hypothesis. Additional refutation is suggested by the failure to recover virus from the spleens of birds which had already recovered from concurrent infections with virus and patent malaria, although it is apparent that such findings could result from the dilution of virus with splenic tissues and fluids. The evidence that concurrent infection of canaries with patent avian malaria and encephalitis virus results in a suppression of virus titer in such birds, suggests again that plasmodia do not serve as a reservoir for the virus.

The data relating the effects of concurrent malaria infection in mosquitoes to their ability to transmit virus is subject to misinterpretation. It was shown that the overall virus transmission rate of mosquitoes concurrently infected with malaria and virus did not differ detectably from the overall transmission rate of those infected with virus only. In the conduct of these experiments, a conscious but not entirely successful effort was made to obtain mosquito lots which had fed on birds with uniform levels of viremia, so that differences in virus transmission rates could be related to the presence or absence of malaria infection in mosquitoes, rather than to virus titers in the infecting birds. The difficulty in obtaining uniform levels of viremia in birds stemmed from the tendency of patent *P. relictum* infections to suppress viremia levels, sometimes even below that necessary to render mosquitoes infective (Barnett, 1955). It should be noted, therefore, that the virus-malaria donor birds used were selected and represent only malaria infected birds with viremia levels above the minimal threshold level of mosquito infectivity. If a larger series of experiments had been

conducted with lots of mosquitoes which had fed on unselected birds, a significant difference in virus transmission rates might have occurred. Such a difference would have to be attributed to the occurrence of lower viremia levels in malaria infected donor birds rather than to the effects of malaria parasitization on the mosquitoes *per se*. The possible importance of such a phenomenon in the epidemiology of Western equine encephalitis is difficult to assess, but it is of interest to note that Herman *et al.* (1954) reported that the incidence of malaria parasitemia in English sparrows and house finches is highest during the months of June, July and August in Kern County, California. Thus, the period of maximal avian malaria infection in a bird population coincides with the period of maximal virus dissemination in the same area (Hammon *et al.* 1945a). It would be of considerable interest to determine if annual prevalence rates of avian malaria in bird populations reflect themselves inversely in the annual occurrence of virus infected mosquitoes.

SUMMARY

A report is presented of the results obtained in a study designed to test the hypothesis that avian malaria plasmodia acquire and retain, for considerable lengths of time, the virus of Western equine encephalitis in infected birds. Experiments were performed to determine the virus concentration of plasmodia-infected blood cells from canaries concurrently infected with both agents and in blood cells from canaries infected with the virus only. The duration of viremia in birds suffering concurrent infection with both agents and in canaries infected with virus only was also determined. These and other studies failed to support the stated hypothesis. Quite the contrary, it was found that concurrent infection of canaries with avain malaria and with encephalitis virus resulted in a significant suppression of virus titer in such birds. This virus titer suppression could not be correlated with the degree of parasitemia, and did not occur in birds in the latent stage of malaria infection.

Concurrent infection of the mosquito *Culex tarsalis* with the avian malaria parasite *Plasmodium relictum* and Western equine encephalitis virus did not detectably alter the ability of this mosquito to transmit virus. Mosquitoes with such concurrent infections transmitted virus in 42 per cent of all attempts, whereas mosquitoes infected solely with virus transmitted in 45 per cent of all attempts. It was pointed out, however, that results of viremia studies on canaries, indicate that concurrent infection of such birds with the two agents suppresses the level of viremia, and that some birds develop virus titers too low to render mosquitoes infective. The possible importance of this phenomenon in the epidemiology and epizootiology of Western equine encephalitis is discussed.

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HISTOPLASMIN SENSITIVITY IN INDONESIA[1]

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About ten years ago it was recognized that histoplasmosis occurs not only as a rare, often fatal, systemic disease but also—and far more frequently—as a benign, asymptomatic infection that may result in pulmonary infiltration and calcification. Large-scale epidemiological surveys in the United States had revealed that up to 80 per cent of the population in some areas were sensitive to skin testing with histoplasmin and that in such areas X-ray evidence of pulmonary calcification was much more frequently associated with histoplasmin sensitivity than with tuberculin sensitivity (Christie and Peterson, 1945; Palmer, 1945). Since then, histoplasmin surveys have been carried out in many countries and high frequencies of histoplasmin sensitivity have been encountered in some areas outside the United States (Mochi and Edwards, 1952; Edwards and Kiaer, 1956).

In Indonesia no histoplasmin testing had been done until quite recently, though clinical cases of histoplasmosis were by no means unknown in this country. Three cases have been reported in life-time residents of Java[3], the first in 1932 (Müller, 1932), the second and third in 1949 (Bras *et al.*, 1949; Hausman and Hiemstra, 1949); and a further three cases have been diagnosed in Europeans returning home after many years of residence in Indonesia (Kirsch and Fressel, 1952; Dowe *et al.*, 1953; Schultz, 1937). Early in 1953, a series of surveys was started to obtain information on the prevalence of histoplasmin sensitivity in Indonesia and its relation to X-ray evidence of pulmonary calcification. Histoplasmin and tuberculin testing, supplemented by X-ray examination of the chest, has now been carried out in over 2,500 persons in the city of Djakarta (Java) between April, 1953, and September, 1954. The work was organized and conducted by the Department of Parasitology and General Pathology of the University of Indonesia, in cooperation with the WHO Tuberculosis Research Office, Copenhagen.

Study population. Pertinent facts concerning the groups of persons included in the present study are summarized in Table 1. At the time of the testing, they were all living in Djakarta. However, many of them had come to Djakarta from different parts of the country, most of them from Java and Sumatra.

A total of 2,542 persons were available for testing in the six study groups.

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[3] A fourth case has recently been diagnosed by culture in a life-time resident of Indonesia and will soon be published by Lie Kian Joe and his associates.

TABLE 1

Information on combined histoplasmin, tuberculin and chest x-ray survey of 6 population groups in Djakarta, 1953–54

Group no.	Character of study group	Date	Age-range	No. in study group	No. with histoplasmin test read	No. with tuberculin test read	No. with both tests read	No. with both tests and chest film read	Tuberculin test		Histoplasmin test	
									Dose	Product	Dilution	Product
			years									
1	Students, University of Djakarta	Sept.–Oct.'53	16–32*	795	714	714	714	704	3 TU	RT XIX-XX-XXI	1:100	H-42
2	Patients, general hospital	April–May '53	1–70	267	256	235	232	185	3 TU	RT XIX-XX-XXI	1:100	H-42
3	Nurses	July–Aug. '53	16–52†	458	445	445	445	443	3 TU	RT XIX-XX-XXI	1:100	H-42
4	Children, elementary school	April '54	9–18	422	418	414	414	411	5 TU	PPD 119, 127, 141	1:100	H-42
5	Children, elementary school	June '54	4–14	335	320	320	320	314	5 TU	PPD 119, 127, 141	1:100	H-42
6	Children, elementary school	Aug.–Sept. '54	4–14	265	258	258	258	254	5 TU	PPD 119, 127, 141	1:100	H-42
Totals...				2,542	2,411	2,386	2,383	2,311				

* 95% fall within the age-range 19–26 years

† 95% fall within the age-range 17–29 years

Both histoplasmin and tuberculin tests were given and read in 2,383 (93.7%); and both tests and a chest film were read in 2,311 (90.9%). On an average only 6 per cent failed to complete the tests and a further 3 per cent either had no chest film taken or the film was technically unsatisfactory for reading.

The first three groups were adults (except for a very small number of children among the general hospital patients); the last three were schoolchildren. All groups included both males and females and some, though predominantly Indonesian, included persons of other racial background.

Technique. All persons received two simultaneous tests, one with histoplasmin and one with tuberculin. The tests were given by injecting 0.1 ml. of diluted antigen intradermally in the volar aspect of the forearm. Throughout each survey one arm was uniformly used for the histoplasmin test and the other for the tuberculin test.

Both tests were read at 48 hours, the transverse diameter of palpable induration being measured in millimeters; no attempt was made to classify the reactions as positive or negative. To ensure uniformity the same person read all the reactions.

For the histoplasmin testing 1:100 dilutions of lot H-42[4] were used. The tuberculin testing was carried out in groups 1–3 with 3 TU dilutions of PPD tuberculin batch RT XIX-XX-XXI (Statens Seruminstitut, Copenhagen); a second test with 30 TU was given to persons with reactions of less than 5 mm. to the 3 TU test. Groups 4–6 were tested with a single dose of 5 TU of PPD tuberculin—batch numbers 119, 127 and 141—supplied by the Public Health Research Laboratories, Manila.

Syringes and needles used for injecting tuberculin were kept strictly separate from those used for histoplasmin injections.

Survey (70 mm.) chest films were taken of most persons and read for infiltrations and calcifications by one experienced reader who had no knowledge of the skin test results.

RESULTS

In presenting the results, the three adult groups (university students, hospital patients and nurses) will be shown separately and the three groups of schoolchildren combined into one group, as little difference was found among them.

Histoplasmin sensitivity. The results of the histoplasmin testing are shown in Figure 1 as frequency histograms by size of induration of the reactions for each of four groups. In the adult groups about 75 per cent of the persons tested had negligible reactions (0–1 mm.), a small proportion had reactions measuring 2–5 mm. and the remaining 10–12 per cent had sizable reactions that tend to form a more or less distinct group centering around 10–11 mm. in size and ranging from 6–20 mm. These moderate-to-large-sized reactions can appropriately be regarded as "positive", the smaller reactions measuring less than 6 mm. as "negative".

[4] For groups 1–3 ready-made 1:100 dilutions were sent by air from the WHO Tuberculosis Research Office, Copenhagen; for groups 4–6 dilutions were made in Djakarta from stock solution supplied by the U. S. Public Health Service, Washington, D. C.

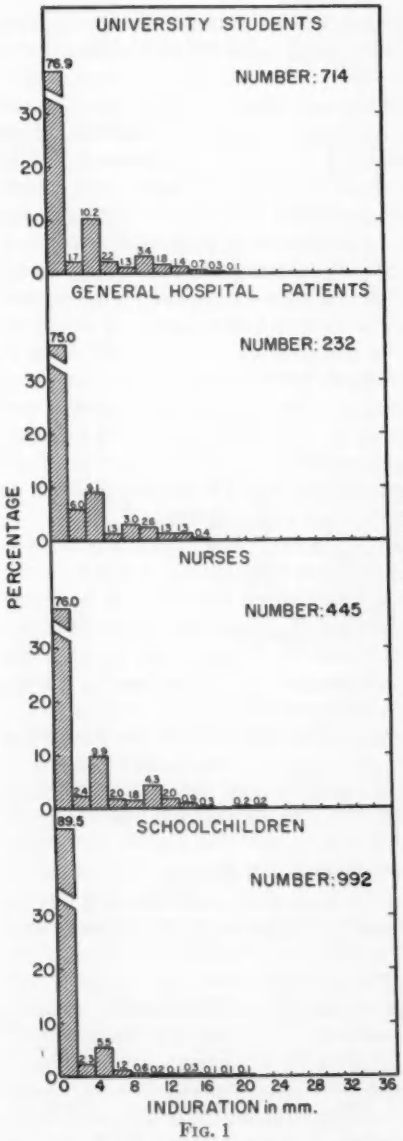

FIG. 1

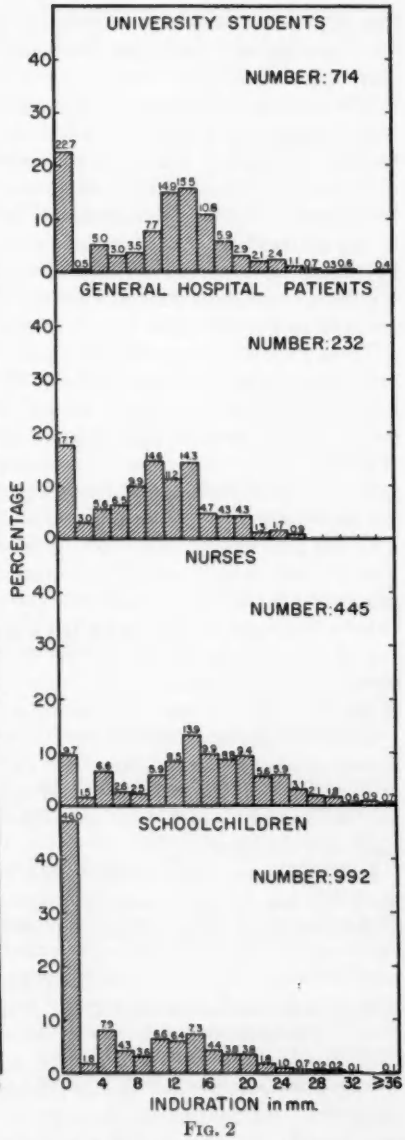

FIG. 2

FIG. 1. Frequency distributions by size of reactions to the intradermal 1:100 histoplasmin test in four population groups, Djakarta, 1953–54.

FIG. 2. Frequency distributions by size of reactions to the intradermal 3 TU test in university students, general hospital patients and nurses, and to the intradermal 5 TU test in schoolchildren, Djakarta, 1953–54.

For the schoolchildren, so few reacted with more than 5 or 6 mm. that a group of "positives" can hardly be distinguished from the vast majority with little or no reaction to the test.

The disproportionately large number of 4–5 mm. reactions in each distribution can probably be ascribed to a reading bias: the reader apparently tended to measure "doubtful" small reactions as either 4 or 5 mm. This interpretation of the results is substantiated by the presence of a similar accumulation at 4–5 mm. for the tuberculin reactions read at the same time (cf. Figure 2). Further analysis of the material will therefore be based on a criterion of 6 mm. or more for a "positive" reaction, to avoid overestimating the percentage of positives, although a criterion of 5 mm. is generally used to distinguish a positive from a negative reaction.

Table 2 gives the percentage of positive histoplasmin reactions, according to sex, in each of the four population groups. In all four groups there is a somewhat higher percentage of positives among the males than among the females. Comparison of the percentage for schoolchildren with those for the adults indicates that the frequency of positive reactions increases with age. Calculation of the rate of increase with age is, however, not feasible with the present material as the percentage of positives is too small to provide stable figures.

Of the four groups, two—the university students and the hospital patients—were biracial in composition including, in addition to Indonesians, a substantial proportion of Chinese. Positive reactions to histoplasmin were found to be nearly twice as frequent among the Indonesian students as among the Chinese students —12.4 per cent as against 7.5 per cent—while the opposite was true for the patients, 16.3 per cent of the Chinese patients having positive reactions compared with only 7.7 per cent of the Indonesian patients.

Reliable residence histories are available for the students and the nurses, the largest number of whom came from Java and Sumatra. The highest percentage of histoplasmin reactors was found among those coming from West Sumatra and the lowest among those from North Java. The differences, however, are small and not significant.

Tuberculin sensitivity. Distributions by diameter of induration of reactions to the 3 TU test for the university students, patients and nurses and of 5 TU reactions for schoolchildren are shown in Figure 2. In each distribution two concen-

TABLE 2

Frequency of positive reactions (6 mm. or more of induration) to the intradermal 1:100 histoplasmin test, by sex, in four population groups, Djakarta 1953–54

Population group	No. with histopl. test read	Per cent with positive reaction		
		Total	Males	Females
Students	714	11.2(80)	12.7	5.4
Patients	256	9.0(23)	9.8	8.0
Nurses	445	11.7(52)	12.8	10.9
Schoolchildren	996	2.7(27)	3.9	1.3

trations of reactions may be seen: one to the left consisting of very small and zero reactions that may be regarded as "negative", and one to the right of fairly large ("positive") reactions centering around 12–16 mm. Although the two concentrations overlap to a greater or lesser degree in each distribution, a separation made between 5 and 6 mm. will give a fair estimate of the frequency of "positives" in each group. As might be expected, the frequency of presumably infected persons is considerably lower in the schoolchildren than in the adult groups: about 45 per cent of the schoolchildren are positive as compared with from 70 to 80 per cent of the adults.

A second tuberculin test with 30 TU was given to 233 of the 294 adults who reacted with less than 5 mm. to the 3 TU test. The total distribution of 30 TU reactions is presented in Figure 3. It appears that a large proportion of those

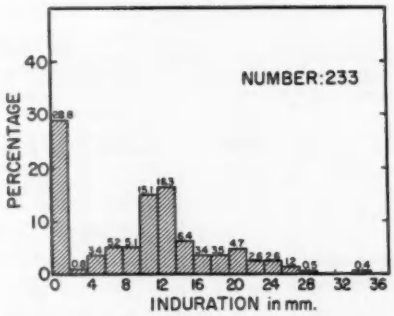

FIG. 3. Frequency distribution by size of reactions to the intradermal 30 TU test in adults reacting with less than 5 mm. of induration to the 3 TU test, Djakarta, 1953–54.

reacting weakly or not at all to the low dose responded strongly to the higher dose. This low-grade sensitivity, which is brought out clearly with a high dose of tuberculin, is very prevalent in many of the Asian countries and is considered "non-specific", i.e., not reflecting tuberculous infection (WHO Tuberculosis Research Office, 1955).

Information on the relation of the histoplasmin test results to the tuberculin test results in the different population groups is given in Table 3. The material shows that 5.3 per cent of the total study population were positive to both histoplasmin and tuberculin while 2.3 per cent were positive to histoplasmin only. About one-third of the total number of histoplasmin reactors were negative to tuberculin, the proportion varying from roughly one-seventh in the patients group to two-thirds in the schoolchildren. This is a fair indication that the histoplasmin had not been contaminated by tuberculin during testing, a not infrequent source of error in combined tuberculin-histoplasmin surveys (Mochi and Edwards, 1952).

X-ray findings. The findings are presented in Table 4 for all persons for whom both tests and an X-ray were read. Out of a total of 2,311 persons, 25 or 1.1 per

TABLE 3

Result of the intradermal histoplasmin and 3 TU (5 TU) tuberculin tests in four population groups

Population group	No. with histopl. test and tuberc. test read	Histopl. test: neg. Tuberc. test: neg.		Histopl. test: pos. Tuberc. test: neg.		Histopl. test: neg. Tuberc. test: pos.		Histopl. test: pos. Tuberc. test: pos.	
		Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
Students	714	177	24.8	24	3.4	457	64.0	56	7.8
Patients	232	58	25.0	3	1.3	151	65.1	20	8.6
Nurses	445	67	15.0	12	2.7	326	73.3	40	9.0
Schoolchildren	992	536	54.0	17	1.7	429	43.3	10	1.0
Total	2,383	838	35.2	56	2.3	1,363	57.2	126	5.3

TABLE 4

Frequency of pulmonary calcifications and infiltrations in total study population according to result of the intradermal histoplasmin and 3 TU (5 TU) tuberculin tests

Result of skin tests	Number with both skin tests and film read	Calcifications		Infiltrates	
		Number	Per cent	Number	Per cent
Histoplasmin test: negative Tuberculin test : negative	811	2	0.2	10	1.2
Histoplasmin test: positive Tuberculin test : negative	56	1	—	0	—
Histoplasmin test: negative Tuberculin test : positive	1,323	17	1.3	49	3.7
Histoplasmin test: positive Tuberculin test : positive	121	5	4.1	6	5.0
Total	2,311	25	1.1	65	2.8

cent had pulmonary calcifications—a lower frequency than that reported from studies in the more temperate zones (Danish Tuberculosis Index, 1955; Goddard *et al.*, 1949; Nissen Meyer, 1951)—and 65 or 2.8 per cent presented X-ray evidence of pulmonary infiltrates. For the adult population—excluding the school-age group—the corresponding percentages were 1.5 and 4.4 respectively. It is seen from Table 4 that the proportion of calcifications and infiltrations was highest in persons who were positive to both tuberculin and histoplasmin; less high in those positive to tuberculin only; and minimal in persons negative to tuberculin whether or not they reacted to histoplasmin.

DISCUSSION

From the material reported here it is evident that histoplasmin sensitivity is at least moderately prevalent in Indonesia: about 10 per cent in each of the 3

adult population groups responded with a sizable reaction to the histoplasmin skin test. As these persons live in an area where cases of clinical histoplasmosis have been diagnosed, there is some ground for believing that the histoplasmin sensitivity reflects infection with *H. capsulatum*. It is known that infections with other fungi, such as *Coccidioides immitis* and *Blastomyces dermatidis*, may produce cross-reactions with histoplasmin; however no cases of disease caused by these other fungi have yet been diagnsoed in Indonesia, so far as is known.

Of some significance is the question whether in a given region pulmonary calcifications (and infiltrates) may be taken as a reliable sign of tuberculous infection or whether other pulmonary infections such as histoplasmosis must be considered in the differential diagnosis. The relatively small number of pulmonary calcifications in the present material necessarily limits our conclusions in this respect. As might be expected in a population in which tuberculosis is highly prevalent, X-ray evidence of pulmonary calcifications was, with few exceptions, found among persons positive to tuberculin. Yet the proportion of calcifications was higher among those positive to both tuberculin and histoplasmin than among those positive to tuberculin only—4.1 per cent compared with 1.3 per cent.

The present study of histoplasmin sensitivity and its relation to pulmonary calcifications demonstrates, we believe, the need for further surveys with a wider geographic coverage of Indonesia. It may well be that the frequency of histoplasmin reactors is considerably higher in some parts of the country than in others and it would be important to locate endemic foci, if such exist. More detailed studies are also needed before we can say with reasonable certainty whether or not histoplasmosis is a disease we have to contend with in Indonesia. The results so far suggest that perhaps it is.

SUMMARY

To obtain information on the prevalence of histoplasmin sensitivity in Indonesia and its relation to radiographic evidence of pulmonary calcifications, a preliminary survey including simultaneous skin-testing with histoplasmin and tuberculin and X-ray examination of the chest was carried out in Djakarta Java in 1953–54. The study population comprised a total of 2,542 persons—795 university students, 267 patients in a general hospital, 458 nurses and 1022 schoolchildren.

The survey results indicate that skin sensitivity to histoplasmin is at least moderately prevalent in Indonesia; 2.7 per cent in the school-age group and from 9 to 12 per cent in the adult groups had sizable reactions to histoplasmin (6 mm. or more of induration). In all four groups the percentage was somewhat higher for the males than for the females. Among the nurses and the students those coming from West Sumatra had the highest percentage of positive histoplasmin reactions and those coming from North Java the lowest; the difference, however, was not very marked. The simultaneous tuberculin test was negative in about one-third of the histoplasmin reactors.

Out of a total of 2,311 persons X-rayed, only 25 or 1.1 per cent had pulmonary

calcifications. For the adult groups the corresponding percentage was 1.5. Almost all of the calcifications were found in persons positive to tuberculin, yet the proportion of calcifications was higher among those positive to both tuberculin and histoplasmin than among those positive to tuberculin only. This suggests that histoplasmosis as well as tuberculosis is a cause of pulmonary calcification in Indonesia.

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STUDIES IN SHIGELLOSIS. V. THE RELATIONSHIP OF AGE TO THE INCIDENCE OF *SHIGELLA* INFECTIONS IN EGYPTIAN CHILDREN, WITH SPECIAL REFERENCE TO SHIGELLOSIS IN THE NEWBORN AND IN INFANTS IN THE FIRST SIX MONTHS OF LIFE[1]

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Although it is well established that children in general are susceptible to bacillary dysentery, there is evidence that the incidence of infection with *Shigella* organisms varies between different age groups of children. In particular, *Shigella* infection rates in infants in the first year of life have been reported to be significantly lower than those found in older children (Blacklock and Guthrie, 1937; Bojlen, 1934; Charles and Warren, 1929; Cooper *et al.*, 1939; Feig, 1951; Felsen, 1945; Hardy and Watt, 1944, 1948; Leibovitz, 1950; Levinson and Raycraft, 1950; Macumber, 1942; McGinnes *et al.*, 1936; Rauss *et al.*, 1953; Watt and Hardy, 1945; Weil, 1943; Williams, 1951). However, with the exception of the investigations of Hardy and Watt and of McGinnes and co-workers, most of the data on this aspect of shigellosis have been derived from consecutive clinical cases, usually severe enough to have required hospitalization. In view of the high percentage of subclinical and asymptomatic *Shigella* infections known to occur in more general samples of the population, this type of data may not be entirely representative. Furthermore, it is difficult to evaluate differences in infection rates among subjects possibly under very dissimilar conditions of exposure.

As pointed out by Felsen (1945), little is actually known about shigellosis in the newborn. Although isolated cases of acute bacillary dysentery in this age group have been reported, the literature, as reviewed by Gordon and Rubenstein (1950), is in general agreement that it is rare. Most investigations have been concerned either with epidemics of diarrhea in institutions or with single acute clinical cases, and no reports have been found of linear studies on *Shigella* infections in more random samples of newborn infants.

Between September, 1951 and August, 1954 a series of studies on shigellosis were made among children in an Egyptian village. By means of weekly bacteriological examinations, correlated with clinical observations during part of the studies, it was possible to follow closely the incidence of *Shigella* infections among representative groups of subjects under five years old for periods as long as one year. The highly endemic environment in which the children lived, and the universal prevalence of *Shigella* infections have been described earlier (Floyd, 1954; Higgins and Floyd, 1955; Higgins *et al.*, 1955a). These conditions provided an

[1] The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the Naval service at large.

unusual opportunity to compare rates of infection, both symptomatic and asymptomatic, between various age groups of children under apparently comparable exposure. In addition, while diarrheal disease has been reported as the cause of over fifty per cent of deaths in Egyptian infants under one year old (Ministry of Finance and Economy, 1952), little is known about the etiology of the illness. It was felt that the presently reported studies might yield useful information on this subject.

MATERIALS AND METHODS

Observations were made among four groups of Egyptian children, totaling 353 individuals, during a three year period between September, 1951 and August, 1954. All subjects lived in Sindbis, a village of 5,000 inhabitants, twenty kilometers from Cairo. Most of the children were utilized in other studies on shigellosis. For reasons of clarity the different groups will be considered separately.

A. *Incidence of* Shigella *infections in children over and under six months of age. 1951–1952.* In a preliminary bacteriological study of the incidence of *Shigella* infections, weekly rectal swab cultures were made on 75 children from October, 1951 through September, 1952. Selection of subjects, methods of observation and over-all results have been reported earlier (Floyd, 1954). These children were between one week and three years old. For the purposes of the present study they were divided into two groups, those over and under six months of age at the start of the investigation. There were 41 in the older group, with a mean age of 16.5 months, and 34 in the younger group, with a mean age of 2.3 months. Age and sex data are shown in Table 1.

B. *Incidence of* Shigella *infections in children by six months age groups. 1953.* In 1953, during studies on diarrheal disease in children in Sindbis, weekly clinical and bacteriological observations were made on 209 subjects from April through October. Methods of selection and observation in this group have been described in detail by Higgins and Floyd (1955). These children were between one month and five years old, with a mean age of 27.6 months, at the beginning of the study. For comparison of *Shigella* infection rates between different ages they were divided into nine age groups as shown in Table 2.

One hundred of these children received a course of immunization with a *Shigella flexneri 3* vaccine during April and May of 1953. The inclusion of this vaccinated group in the present study was considered valid since it was shown

TABLE 1

AGE AND SEX DATA ON SUBJECTS IN PRELIMINARY STUDY (1951-2).

AGE GROUP	NUMBER SUBJECTS	SEX		MEAN AGE MONTHS
		MALE	FEMALE	
OVER 6 MONTHS	41	22	19	16.5
UNDER 6 MONTHS	34	18	16	2.3
TOTAL	75	40	35	10.1

that immunization did not change the incidence of *Shigella* infections, or of diarrheal disease, among these children (Higgins *et al.*, 1955b). A second 100 were the subjects of an investigation on the incidence and etiology of diarrheal disease and also served as a control group for other studies (Higgins *et al.*, 1955a). The remaining nine, identically selected and observed, were supernumeraries to the latter group.

C. *Incidence of* Shigella *infections in newborn infants. 1953.* Evidence obtained from a small number of newborn infants in the study of 1951–2 indicated that even in a highly endemic area *Shigella* infections were extremely rare in the 30 day neonatal period. For more specific data a group of 28 infants, born in the expected season of highest incidence of shigellosis in 1953, were followed by weekly bacteriological observations during and after the neonatal period. These subjects constituted the majority of live births reported to the Rural Health Center in Sindbis during the last two weeks of June. The recording of the birth and the willingness of the mother to cooperate were the only factors affecting selection.

Weekly cultures were started as soon as infants were admitted to the study. On the average the subjects were 10.8 days old when the first cultures were taken. Observations were continued until the investigation was terminated in October. Bacteriological methods employed were the same as those described in reports cited earlier. It was not possible to make coordinated clinical observations in this group. The 209 older children discussed above, who were being followed by the same methods during this period, served as a control group for this study.

D. *Incidence of* Shigella *infections in newborn infants. 1954.* Additional information was obtained in 1954 from a group of 41 newborn infants who were born between the middle of April and the middle of June in that year. These subjects were obtained from birth reports at the Rural Health Center in Sindbis and from

TABLE 2

AGE DISTRIBUTION OF A GROUP OF 209 EGYPTIAN CHILDREN

AGE GROUP (MONTHS)	NUMBER SUBJECTS	MEAN AGE MONTHS
1 - 6	16	4.0
7 - 12	28	9.0
13 - 18	24	15.8
19 - 24	36	20.7
25 - 30	16	28.0
31 - 36	19	33.8
37 - 42	26	39.0
43 - 48	29	45.7
49 - 62	15	53.1
TOTAL	209	27.6

case finding by nurses and technicians. The selection was influenced only by the ability to discover new births and the willingness of the mothers to cooperate. It was estimated, using birth rates obtained by Weir and co-workers (1952) in the most recent vital statistics survey of this village, that this group of 41 probably represented about 80 per cent of the total births during that period. On the average the subjects were ten days old when admitted to the study.

Weekly clinical and bacteriological observations, as described in the reports cited earlier, were started as soon as an infant was added to the group and continued through the second week in August. Again, this was the expected season of highest incidence of shigellosis in this village.

A group of 102 children, remaining from the 209 in the study during the previous year and being observed by similar methods, served as controls. These children were now between one and six years old.

RESULTS

A. *Isolation of* Shigella *organisms from children over and under six months of age. 1951–1952.* As reported earlier (Floyd, 1954), *Shigella* organisms were isolated from 291 (8.0%) of 3618 rectal swab cultures made on the 75 children observed between October, 1951 and September, 1952. Of these positives, 190 (9.7%) were recovered from 1,946 cultures from subjects over six months of age, and 101 (6%) were from 1,672 cultures from those less than six months of age at the start of the study. When the data are examined by half year periods (October–March, April–September) the results are more striking. As shown in Table 3, during the first six months period *Shigella* organisms were isolated from 41 (4.4%) of 922 rectal swab cultures from the older group while only seven (0.8%) of 829 cultures from the younger children yielded the organisms. During the latter half of the year, when the average age of the younger children was around eight months, there was little difference in the percentages of positive cultures recovered from the two groups.

During the year of observation an average of 3.8 positive cultures were obtained from each child. Subjects in the older group had an average of 4.6 positives while the younger group had 2.9. From October to March the average for the older subjects was 1.0 as compared to 0.2 for the younger. Averages for the second half of the year were 3.6 and 2.7 respectively.

Incidence of Shigella *infections in children over and under six months of age. 1951–1952.* As previously reported, 73 of the 75 children were infected with

TABLE 3

ISOLATION OF SHIGELLA ORGANISMS FROM CHILDREN OVER AND UNDER SIX MONTHS OF AGE. 1951–1952.

AGE GROUP	OCT. 1951 – MAR. 1952			APR. 1952 – SEPT. 1952			TOTAL YEAR		
	NUMBER CULTURES	NUMBER POSITIVE	PERCENT POSITIVE	NUMBER CULTURES	NUMBER POSITIVE	PERCENT POSITIVE	NUMBER CULTURES	NUMBER POSITIVE	PERCENT POSITIVE
OVER 6 MONTHS	922	41	4.4	1024	149	14.5	1946	190	9.7
UNDER 6 MONTHS	829	7	0.8	843	94	11.1	1672	101	6.0

TABLE 4

CHILDREN OVER AND UNDER SIX MONTHS OF AGE WITH SHIGELLA INFECTIONS. 1951-2.

AGE GROUP	NUMBER SUBJECTS	SUBJECTS WITH SHIGELLA INFECTIONS			
		OCT. 1951-MAR. 1952		APR. 1952-SEPT. 1952	
		NUMBER	PERCENT	NUMBER	PERCENT
OVER 6 MONTHS	41	10	24.3	38	92.6
UNDER 6 MONTHS	34	3	8.8	31	91.1

Shigella organisms at least once during the year. Positive cultures were eventually obtained from all 34 in the younger group and from 39 of the older children. By examining the data by half year periods an obvious difference between the two groups is noted. In Table 4 it may be seen that during the first half of the year ten (24.3%) of the older children had one or more *Shigella* infections while only three (8.8%) of the younger group were found to be infected in the same period. In the latter half of the year the percentages of children infected at least once was essentially the same in both groups.

B. *Isolation of* Shigella *organisms from children by six months age groups. 1953.* There were 189 of the original group of 209 children remaining at the end of 32 weeks of observation. Ten had died and ten were dropped from the study for lack of cooperation. Of the possible total of 6,048 rectal swab cultures, 5,508 (91%) were delivered to the laboratory. Clinical observations were made each time a culture was obtained.

Shigella organisms were isolated from 387 (7%) of the specimens. In Table 5

TABLE 5

ISOLATION OF SHIGELLA ORGANISMS FROM CHILDREN IN SIX MONTHS AGE GROUPS. 1953.

AGE GROUP (MONTHS)	NUMBER SUBJECTS	CULTURES FOR SHIGELLA			
		TOTAL NUMBER	NUMBER POSITIVE	PERCENT POSITIVE	AV. NO. POS. PER SUBJECT
1-6	13	318	7	2.2	0.5
7-12	25	746	52	6.9	2.0
13-18	20	598	57	9.5	2.8
19-24	29	825	68	8.2	2.3
25-30	13	395	33	8.3	2.5
31-36	23	689	52	7.5	2.2
37-42	26	762	54	7.0	2.0
43-48	23	658	37	5.6	1.6
49-62	17	517	27	5.2	1.5
TOTAL	189	5508	387	7.0	2.0

are shown the numbers and percentages of positive cultures recovered from children in the different age groups. The lowest percentage (2.2%) of positives was found among children from one to six months old. In comparison to this group there was a three-fold increase in the percentage of positives recovered from the 7–12 months old subjects. The highest percentage (9.5%), found in the 13–18 months old children, was more than four times that of the youngest group. Thereafter, percentages of positives decreased with increasing age of the groups. These differences in percentages were statistically significant by Chi square analysis. The average number of positive cultures per child in the various groups followed a similar pattern.

Due to the isolation of repeated positives from single individuals the total of 387 *Shigella* cultures represented 285 separate infections. With respect to diarrhea only, 121 (42.4%) were symptomatic and 164 (57.6%) were asymptomatic. There was no significant difference in the ratio of symptomatic to asymptomatic infections in the first six age groups. On the average 50 per cent were associated with diarrhea. In the three oldest groups, however, an average of only 33 per cent were considered symptomatic.

Incidence of Shigella *infections in children by six months age groups. 1953.* Of the 189 subjects present throughout the study, 156 (82.5%) had at least one *Shigella* infection during the period of observation. In Table 6 it may be seen that only 46.1 per cent of the 1–6 months old children were infected during this time while the percentages in the other groups ranged from 73.9 to 96.1 per cent. The highest proportion of infected children occurred in the 37–42 months old group.

The cumulative percentage curves of Figure 1, in which the groups over six months of age have been combined, show that more than 60 per cent of the

TABLE 6

CHILDREN IN SIX MONTHS AGE GROUPS WITH SHIGELLA INFECTIONS. 1953.

AGE GROUP	TOTAL NUMBER SUBJECTS	SUBJECTS WITH SHIGELLA INFECTIONS	
		NUMBER	PERCENT
1-6	13	6	46.1
7-12	25	20	80.0
13-18	20	16	80.0
19-24	29	24	82.7
25-30	13	12	92.3
31-36	23	21	91.3
37-42	26	25	96.1
43-48	23	17	73.9
49-62	17	15	88.2
TOTAL	189	156	82.5

7–62 months old subjects had already had at least one *Shigella* infection before the first such infection was observed in the 1–6 months old group. The six children in this group who eventually became infected were from seven to twelve months old at the time the organisms were recovered.

In Figure 2 the percentages of children infected with *Shigella* in the 1–6 and 7–62 months old groups are shown at bi-monthly intervals. The highest incidence in the older group occurred from May to August, whereas the majority of infections among the younger children were observed during August, September and October.

Incidence of diarrheal disease in children by six months age groups. 1953. During the period of observation there were 329 separate diarrheal episodes among the 189 subjects, an average of 1.7 episodes per child. Of these, 121 (36.7%) were associated with the presence of *Shigella* organisms. A comparison of the total number of episodes, the average number per child, and the numbers and percentages associated with *Shigella* organisms between the various age groups is shown in Table 7. The incidence of diarrheal disease was highest among the younger children and decreased progressively with age. Only ten per cent of the episodes among the 1–6 months old children were associated with *Shigella*, whereas from 32 to 47 per cent of the episodes in the older children were related to the presence of the organisms.

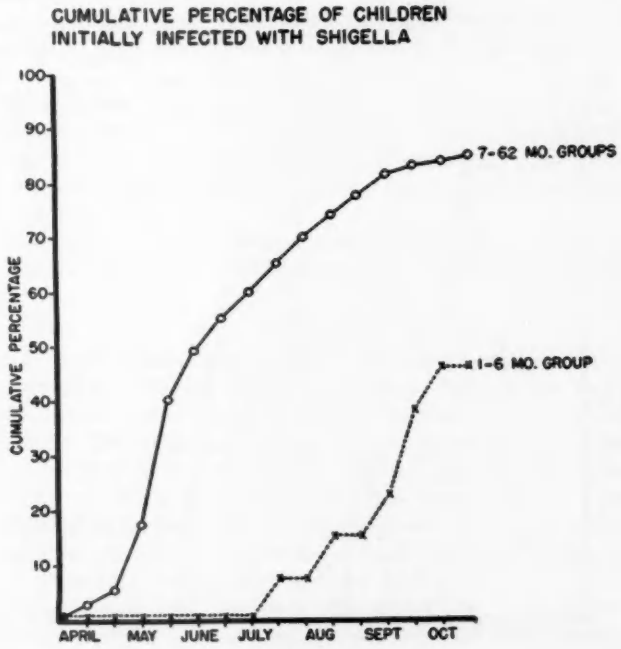

FIGURE 1

C. *Incidence of* Shigella *infections in newborn infants. 1953.* Twenty-one of the original group of 28 newborn infants were present at the conclusion of the study in October, 1953. Four died and three were dropped for lack of cooperation. A total of 369 (88.2%) of a possible 418 rectal swab cultures were obtained. These represented 369 person weeks of observation, 75 of which were made during the neonatal period.

Shigella organisms were isolated from none of the 75 rectal swab cultures from infants in the first month of life. During this same period of time 67 (9.5%) of 702 cultures from the control group of 209 older children were positive for these organisms.

Shigella were recovered from one (0.3%) of 294 rectal swab cultures from the newborn group during the four months of observation following the neonatal period. In comparison, 137 (5.4%) of 2528 cultures from the control group yielded the organisms.

Of a total of 369 cultures, made on 28 infants from birth until they were approximately five months old, one (0.2%) was positive for *Shigella*. During the same period, from the middle of June to the end of October, 204 (6.3%) of 3230 cultures from older controls were positive.

D. *Incidence of* Shigella *infections in newborn infants. 1954.* During 1954, of 41 newborn infants obtained for study shortly after birth in April, May or June,

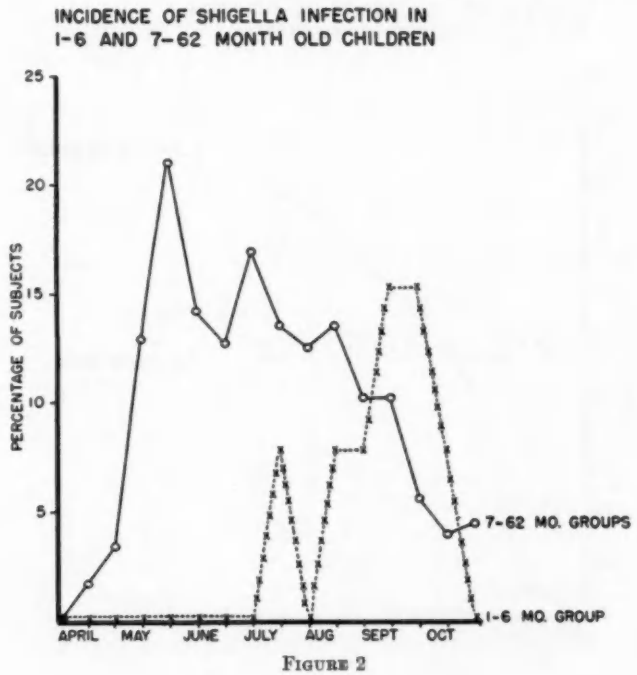

FIGURE 2

TABLE 7

INCIDENCE OF DIARRHEAL DISEASE AMONG SIX MONTHS AGE GROUPS OF CHILDREN. 1953.

AGE GROUP	NUMBER SUBJECTS	DIARRHEAL EPISODES			
		TOTAL NUMBER	AV. NO. PER SUBJECT	ASSOC. WITH SHIGELLA	
				NUMBER	PERCENT
1-6	13	30	2.3	3	10.0
7-12	25	55	2.2	21	38.1
13-18	20	43	2.1	19	44.1
19-24	29	55	1.9	21	38.1
25-30	13	25	1.9	11	44.0
31-36	23	35	1.5	15	42.8
37-42	26	32	1.2	11	34.3
43-48	23	37	1.6	12	32.4
49-54	17	17	1.0	8	47.0
TOTAL	189	329	1.7	121	36.7

36 remained at the conclusion of the study in August. The other five subjects died. There were 429 person weeks of observation, of which 105 were during the neonatal period. Eighty-six per cent of the possible weekly clinical and bacteriological observations were obtained.

Shigella organisms were recovered from none of 105 cultures from neonates. There was one (0.3%) positive among 324 cultures made after the first month of life. During the period from April through August *Shigella* were isolated from 70 (4.5%) of 1,553 weekly rectal swab cultures from the 102 one to six year old children serving as controls.

Diarrhea was recorded in 10.1 per cent of the weekly clinical observations among the newborn group as compared to 7.4 per cent in the older children.

DISCUSSION

The results presented here are further evidence of a relationship between age and the incidence of *Shigella* infections in children. It is felt that they are of particular interest in view of the highly endemic environment and the linear nature of the studies. The findings were generally in agreement with those of earlier investigators (Blacklock, *et al.*, 1937; Charles and Warren, 1929; Cooper, *et al.*, 1939; Hardy and Watt, 1944; Macumber, 1942; among others) in that children under one year of age had relatively fewer *Shigella* infections than older subjects. In Egyptian village children, however, it would appear that this period of low incidence of infection was limited mainly to the first six months of life. This fact was demonstrated in both studies of 1951–2 and 1953 when children approximately 7–12 months old were found to have about the same incidence of infection as older children. Furthermore, by observing a group of children throughout

their first year of life and comparing them with older subjects, it was possible to show an increase in infection rates in the same children as their age increased.

The complete absence of shigellosis in infants in the neonatal period during seasons of high incidence of the disease was noteworthy. Admittedly methods employed for recovering the organisms have limitations, but the fact that 180 person weeks of observation were involved in this finding would indicate *Shigella* infections were extremely rare in this age group.

Diarrheal disease was more prevalent in neonates and in infants under six months of age than in older children, but the rarity with which it could be associated with *Shigella* pointed out the relatively minor etiological role of these organisms in these episodes. The possible causal role of certain serological types of *Escherichia coli* or of viral agents, as has been reported in other parts of the world, can only be speculated upon.

It is usually thought that clinical disease produced by *Shigella* in children in the first few months of life is apt to be severe. Too few such infections occurred in the present study to draw definite conclusions, but it was interesting to note that the three observed in the 1–6 months old group in 1953 were all considered to have been clinically mild.

The period of greatest incidence of *Shigella* infection was found during the second and third years of life, which was in keeping with the observations of Feig (1951) and of Hardy and Watt (1948). After this age relatively fewer infections were noted and there were indications that a larger proportion of infections in children over three tended to be nondiarrheal in nature than were noted in younger groups. As pointed out by Watt (1951), it is difficult to determine whether this may have been due to immunity acquired from previous infections.

No direct evidence was obtained as to whether the low incidence of *Shigella* infections in neonates and in infants in the first few months of life was due to natural resistance or was the result of less exposure to infection than older children. Passive congenital immunity, known to occur in other bacterial infections and to wane around the sixth month of life (Wilson and Miles, 1946), would seem to be a possibility in infants whose mothers have long been exposed to *Shigella* organisms. Felsen and Osofsky (1937) were unable to demonstrate transplacental transfer of significant levels of agglutinating antibodies against *Shigella*, but it is debatable whether these antibodies are correlated with active or passive immunity to the infection (Cooper *et al.*, 1949; Watt and DeCapito, 1945).

The viewpoint has been expressed that infants are less exposed to infection both from the standpoint of food (i.e., breast-feeding) and from other recognized routes of transmission (Blacklock *et al.*, 1937; Charles and Warren, 1929; Felsen, 1945; Ross and Dawes, 1954; Weil, 1943, 1947). This must also be considered as a possibility since it is a general impression that Egyptian village infants are breast-fed, when possible, well into the second year. Supplementary feeding of solid food usually is not started until around the sixth month. Thus the results of the present study may well demonstrate the "epidemiological dividing line" (Weil, 1947) between the infantile diarrheas of uncertain etiology of breast-fed babies and the food-borne shigellosis of older children. However, considering the

constant opportunities for contact with the organisms from sources other than food and the high prevalence of diarrheal disease, some of which was possibly due to agents with routes of transmission presumably similar to those of *Shigella*, it is difficult to ascribe the lower incidence of shigellosis in children under six months of age to this factor alone. A combination of the two factors would appear to be the most likely explanation.

SUMMARY

Egyptian village children in the first six months of life, while exposed to a highly endemic environment and experiencing a higher incidence of diarrheal disease, were less frequently infected with *Shigella* organisms than were older children. *Shigella* organisms were not recovered from infants in the neonatal period even during seasons of high incidence of infection in older subjects. *Shigella* infections were most prevalent during the second and third years of life.

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A METHOD FOR THE ISOLATION OF *SALMONELLA TYPHI* FROM BLOOD CLOT

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Isolation of *Salmonella typhi* from the blood stream is usually regarded as being the most satisfactory method of early diagnosis of typhoid fever (Manson-Bahr, 1950). The introduction of specific antibiotic therapy has made it imperative that the diagnosis should be achieved quickly. The isolation of *S. typhi* from stools and urine during the second and subsequent weeks of illness is not satisfactory from this point of view. Similarly, the observation of rising agglutinin titers later in the illness is not altogether satisfactory since valuable time may be lost in starting treatment if such observations are awaited.

Conventional methods of whole blood culture leave much to be desired, particularly regarding the duration of incubation of cultures which is often required before a positive isolation is achieved. Shaw & MacKay (1951) report an average duration of 4 to 5 days in a series of 71 positive cultures, and 17 per cent of these only became positive on the ninth to eleventh days of incubation. In our experience of 90 positive cultures the average duration of incubation required was 4.8 days with a variation of 1 to 16 days. We have shown that the presence of serum factors in whole blood cultures plays a part in this long incubation period (Watson, 1954).

We have previously reported our findings with blood clot cultures using 0.5% taurocholate broth containing 100 units/ml. of streptokinase as culture medium (Thomas, Watson & Hewstone, 1953). Subsequent experience with this medium has shown that culture of blood clot is more successful than culture of whole blood. The streptokinase breaks up the clot quickly and releases trapped organisms into the medium.

The method reported here has enabled us to obtain positive isolations in less than twenty-four hours.

METHOD

Fifteen ml. of melted Wilson & Blair's bismuth sulphite agar medium are poured into four-ounce sterile bottles. The bottles are placed on their sides and the agar allowed to set. They are then stored at 4°C. till required. For use, 15 ml. of streptokinase[1] bile salt broth (containing 100 units/ml. of streptokinase) are added to each bottle. The residual clot left after removal of the expressed serum from 5.0 ml. of blood is added to the streptokinase broth. Bottles are then incubated at 37°C. for six hours. At the end of this period the broth is allowed to run over the surface of the slope by tilting the bottle for two minutes. The containers are then reincubated and examined after 12 and 24 hours.

[1] The streptokinase used in this investigation was kindly supplied by Messrs. Lederle, American Cyanamid Co.

RESULTS

Growth of *S. typhi* on these slopes usually manifests itself as a thin diffuse film showing the characteristic blackening and sheen. Growth is usually most obvious at the junction of the fluid medium with the slope and is best seen by looking at the slope edge on. Single colonies were only found in those cases with a low grade bacteremia. In a series of fifty artificially infected clots, each containing approximately twenty five viable organisms, growth was evident in each instance in 18 to 24 hours. We have used the method extensively in clinical cases with satisfactory results, and the saving in time as compared with fluid cultures followed by plating out has enabled us to institute therapy more rapidly than was formerly the case. In each instance the growth on the agar slope has proved satisfactory for checking biochemical and serological reactions.

DISCUSSION

Rapid isolation of *S. typhi* from the blood is a matter of some importance and any simple method which will achieve this will have a lot to commend it. Particularly is this so in the case of an epidemic outbreak where early diagnosis and treatment are essential. The method described can often give a positive result in about 18 hours and presents definite advantages over conventional methods of whole blood culture.

SUMMARY

A method for rapid isolation of *S. typhi* from blood clot is presented. The medium used consists of a Wilson & Blair agar slope in a four-ounce bottle to which is added 15 ml. of streptokinase bile salt broth. Positive results may be obtained in less than 24 hours.

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THE DRIED FILTER PAPER TECHNIQUE FOR SENDING STOOL SPECIMENS TO A LABORATORY FOR BACTERIOLOGIC EXAMINATION

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Sending stool specimens to a distant laboratory for bacteriologic identification has always been unsatisfactory. The dried filter paper technique has proved to be an efficient, simple and cheap solution of the problem.

TECHNIQUE

On a clean piece of filter paper 6 to 10 drops of stool are placed and dried at room temperature. For dysenteric stools the mucous parts are selected. A firm stool should be mixed with water to make it semiliquid before dropping it on the filter paper. To obtain satisfactory results the following procedure should be performed:

1. The stool should be dried as soon as possible after defecation.
2. It is essential to dry thick fecal drops; thin drops are unsatisfactory. To reduce the drying period to not more than three hours thick drops should be made having a diameter of about 0.5 cm. consisting of about 0.2 cc. of mucus or stool. The drops should be separated about 5 cm. from each other to allow the moisture to be absorbed by the surrounding paper.
3. The preparations should be dried in a cool place; the lower the temperature, the better the results.
4. The thoroughly dried stools can be sent to a laboratory by airpost. To prevent contamination of the envelope the filter paper should first be sealed in a cellophane envelope. Damp fecal drops give poor results.

Method of culturing stools dried on filter paper. Cut out some of the dried fecal drops and put each sample into 1 or 2 cc. of saline or broth. After 15 to 20 minutes the stools are restored to proper consistency. This should be directly plated on the culture media. To compensate for the loss of bacteria by drying, the amount of stool applied to the culture media should be much more than in the case of a fresh stool examination. The amount of stool used for culture should be in proportion to the length of time it was in the dry state. For example, for stools kept dry for 3 to 5 days the amount to be applied on the media is 10 to 15 times the usual amount. The usual media employed for the cultivation of enteric bacilli such as Endo-agar, McConkey agar, SS-agar, desoxycholate-citrate agar and bismuth sulfite agar can be used for the cultivation of dried stools.

Mortality of bacteria in dried stools. Contrary to the general belief, enteric bacilli usually resist drying better than most nonpathogenic bacteria. The mortality of different *Shigella* strains in stools kept dry for 1 day varies from 60 per cent to 95 per cent and for *Salmonella* from 60 per cent to 90 per cent. *Shigella*

may remain viable in dried stools for as long as 2 months and *Salmonella* up to 6 months. On the other hand most nonpathogenic bacteria resist drying poorly. Their mortality in stools kept dry for 1 day is usually over 90 per cent and they usually succumb after 3 weeks.

RESULTS

The technique described, when performed at room temperature varying from 27°C. to 33°C., gave satisfactory results as compared with tests on fresh stool samples from the same specimen.

Cultures made from dysenteric stools kept dry for 3 days gave 22 per cent less positive results than those obtained by culturing the freshly passed stools. With specimens kept dry for 5 days positive results were about 40 per cent less than with fresh specimens, and with 7-days-old dried stools the positive results were 45 per cent less. These results were obtained from tests on 65 positive dysenteric stools (Soeprapti Kartanegara *et al.*, in press).

Cultures made from typhoid and paratyphoid stools kept dry for 3 days gave 14 per cent less positive results than those obtained by culturing samples from the same freshly passed stools. The number of positive results became 17 per cent less when the specimens were kept dry for 5 days. These results were obtained from a study with 42 positive typhoid and paratyphoid stools (Lie Kian Joe *et al.*, 1954).

The technique as applied to dysenteric stools compared favorably with the buffered glycerolsaline preservative and with the sodium desoxycholate-citrate solution as recommended by Bangxang and Eliot. Twenty-six positive dysenteric stools were partly dried and also partly stored in the above mentioned preservatives. They were then cultured on the 3rd and 5th day. On the 3rd day the dried stool specimens gave 76 per cent positive results and on the 5th day 68 per cent. The glycerol-saline specimens gave 73 per cent on the 3rd day and 35 per cent positive results on the 5th day, while the results obtained from the use of sodium desoxycholate-citrate fluid were respectively 46 per cent and 23 per cent.

DISCUSSION

The dried filter paper technique was satisfactory for tests made locally as well as for tests on specimens sent to distant laboratories (Lie Kian Joe, 1950 a and b, 1954; Beeuwkes 1952; Wolff 1954; Bonebakker, 1954; Hulshoff, 1954; and Bijlmer, 1954).

However, Kirsche and Prévot (1953) applying the drying method to dysenteric stools obtained poor results with only 28.4 per cent positive results from 1-day-old dried stools and 6.1 per cent from stools kept dry for 3 days. Their technique of drying may have been the cause. They soaked the filter paper in dysenteric stools instead of making small, thick fecal drops. Soaking a filter paper in dysenteric stools gives usually only a thin, unsatisfactory film. Moreover, it does not provide for selection of the mucus where the *Shigella* are usually concentrated.

The mortality rate among nonpathogenic bacteria in dried stools usually is

higher than among enteric bacilli. This would result in a favorable change of the ratio between pathogenic bacteria growing on the culture media. This relative "enrichment" in the number of pathogens was observed in more than 50 per cent of positive typhoid stool cultures (Lie Kian Joe, 1954) and also can be observed in dysenteric stool cultures though not in such a high proportion. Moreover, pathogenic bacteria usually outlive nonpathogenic bacilli in dried stools, and pure cultures of pathogenic bacteria are not infrequently obtained from dried stools.

The temperature has great influence on the survival of bacteria in dried stools. The lower the temperature under which the stools are dried and stored, the better are the results. The mortality among bacteria is high when the stools are dried at 37°C. It is therefore to be expected that the dried filter paper technic will give poor results in areas where the room temperature is 37°C. or higher.

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ON THE INFECTIVITY OF THE FORMOSAN STRAIN OF *SCHISTOSOMA JAPONICUM* IN MACAQUES[1]

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Results of previous studies on the epidemiology of schistosomiasis japonica in Taiwan (Formosa) indicated that there is a non-human or zoophilic strain of *Schistosoma japonicum* (Hsü *et al.*, 1952, 1954, 1955). Experimental exposure of human volunteers would have given final proof of this. However, before such an attempt was to be made, it was thought desirable to make a preliminary study on the infectivity of this strain in monkeys. The present paper gives the results of experimental infection of the Formosan strain of *S. japonicum* in the following monkeys: *Macacus cyclopis* from Formosa, *M. fuscatus* from Japan, and *M. philippinensis* from the Philippines. For comparison, Formosan monkeys were exposed to human strains of the parasite from Japan and the Philippines.

MATERIALS AND METHODS

Cercariae of the Formosan strain of *S. japonicum* were obtained from the snail, *Oncomelania formosana*, collected in the Changhua endemic area. The Japanese strain was obtained from *O. nosophora* collected in the Kyushu area, and *O. quadrasi* from Leyte served as the sources of the Philippine strain.

The three species of monkeys used in the experiments were purchased from animal dealers in Taipei, Tokyo and Manila. No individual information or history could be obtained for these animals. The ages, classified as young, young adult, adult and old in this paper, were estimated by the size and appearance only. A total of 36 monkeys (29 from Formosa, 3 from Japan, and 4 from the Philippines) were used in this study. The sedimentation technique was used in the examination of the stools from each monkey over a 3-day period. All of the animals used were negative for *S. japonicum* eggs.

The cercariae used in the infection of each monkey were pooled from 10 infected snails. Each monkey was exposed by placing about 600 mature cercariae on the skin. Concurrently albino mice or dogs were used as control animals in each experiment in order to assure the infectivity of the cercariae as well as the bisexual composition of the cercarial mixture. All the control animals were found to produce *S. japonicum* eggs.

Daily stool examination by sedimentation was started on all the monkeys 28 days after the exposure. Five grams of feces were used in each examination and the last 0.4 ml. of the sediment, divided on 4 slides, was examined for *S. japonicum* eggs. The number of immature, mature and degenerate eggs observed

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in these samples was recorded. When a monkey died or was sacrificed, a careful examination was made of the mesenteric and portal veins for worms. In certain cases, mucosa of the intestinal wall was scraped and a portion of liver was digested in KOH solution to facilitate the examination of *S. japonicum* eggs.

Five series of experiments were done and each one was assigned an appropriate abbreviation: F-F for Formosan monkeys exposed to the Formosan strain of *S. japonicum*; F-J for Formosan monkeys exposed to the Japanese strain; F-P for Formosan monkeys exposed to the Philippine strain; and J-F for Japanese monkeys exposed to the Formosan strain. For the sake of brevity these abbreviations have been used throughout the paper.

RESULTS OF EXPERIMENTS

F-F Series. Altogether 19 Formosan monkeys were exposed to cercariae of the Formosan strain of *S. japonicum*. One of these was a young adult (No. 25) and all of the others were adults. *S. japonicum* eggs were obtained in the stool examination of 13 monkeys and the other six were negative. All of the monkeys in the latter group died or were killed 27 to 70 days after exposure. It will be noted in

TABLE 1

Results of stool examinations and autopsy findings of 19 monkeys in the F-F series

Monkey No.	Prepatent period (Days)	Egg count period: Begin-End (Days after infection)	Maximal daily egg production	Average daily egg production in positive stools (1)			Termination (Days)	Worm finding at autopsy			
				Immature	Mature	Degenerate		Portal vein and its branches		Mesenteric veins	
								M	F	M	F
1	—	—	—	—	—	—	55, killed	2	1	0	0
2	—	—	—	—	—	—	70, killed	0	0	0	0
3	—	—	—	—	—	—	70, killed	0	0	0	0
4	—	—	—	—	—	—	50, killed	1	1	4	4
5	88	88–365	6	0.8	0.1	0.2	580, killed	0	0	0	0
6	98	98–174	7	—	—	—	294, killed	0	0	0	0
7	—	—	—	—	—	—	45, died	0	0	0	0
8	93	93–96	1	—	—	—	103, killed	1	0	0	1
13	54	54–235	72	7.3	0.6	0.6	476, living	—	—	—	—
14	35	35–96	1,971	307.4	135.6	86.3	97, died	82	0	142	76
15	37	37–405	4	1.0	0.1	0.2	476, living	—	—	—	—
16	38	38–57	1	—	—	—	191, killed	0	0	0	0
24	51	51–245	13	0.8	0.7	0.6	340, living	—	—	—	—
25	—	—	—	—	—	—	27, died	4	2	0	0
26	40	40–256	1	0.5	0.5	0.0	274, died	0	0	0	0
27	96	96–262	2	0.4	0.3	0.7	337, living	—	—	—	—
28	93	93–223	4	0.6	0.6	0.6	337, living	—	—	—	—
29	82	82	1	1.0	0.0	0.0	131, living	—	—	—	—
30	56	56–273	6	0.8	0.9	0.8	337, living	—	—	—	—

(1) The egg-stage differential counts for monkey No. 5 was started 82 days after the prepatent period; for No. 13, 8 days; for No. 14, 41 days; and for No. 15, 70 days. Egg-stages were not differentially counted for Nos. 6, 8 and 16.

TABLE 2

Results of stool examinations and autopsy findings of 8 monkeys in the F-J series and 2 in the F-P series

Monkey No.	Pre-patent period (Days)	Egg count period: Begin-End (Days after infection)	Maximal daily egg production	Average daily egg production in positive stools			Termination (Days)	Worm finding at autopsy			
				Immature	Mature	Degenerate		Portal vein and its branches		Mesenteric veins	
								M	F	M	F
F-J Ser.											
9	38 (1)	—	—	—	—	—	55, died	38	38	9	4
10	38	83–84 (2)	8,882	—	—	—	112, died	38	38	47	50
31	36	36–69	1,346	27.0	186.2	40.1	69, died	10	7	51	54
32	36	36–250	1,015	5.5	26.0	8.2	250, living	—	—	—	—
33	37	37–242	207	2.1	4.4	2.6	250, living	—	—	—	—
34	36	36–62	1,309	21.3	276.5	24.0	62, died	49	39	62	51
35	36	36–93	350	9.4	54.6	5.0	93, died	49	24	34	17
36	39	39–121	668	7.2	37.3	7.7	121, died	19	8	39	23
F-P Ser.											
11	34	131–148 (2)	1,606	265.9	418.0	57.4	148, died	86	70	9	9
12	35	82–83; 139–466 (2)	1,010	6.5	6.5	6.1	531, killed	1	1	0	0

(1) Egg counts were not made for monkey No. 9, but it was noted that the daily egg production was high and there were more mature eggs than immature ones.

(2) In monkeys Nos. 10, 34 and 35, egg counts were not made throughout the experimental period but only for a limited number of days as indicated. For monkey No. 10, stages of the eggs were not differentially counted but it was noted that the proportion of mature eggs was higher than that of the immature ones. For monkey No. 12, egg-stage differential counts were not done on the 82nd and the 83rd days but it was also noted that there were more mature eggs than immature ones these two days.

Table 1 that many of the other monkeys did not have positive stools until later in the course of their infection. Upon autopsy three of these monkeys (Nos. 2, 3 and 7) were not infected. Two other monkeys (Nos. 1 and 25) harbored immature worms and No. 4 had a pair of mature worms in the mesenteric vein and eight immatures in the portal vein.

The results of stool examinations of the other 13 monkeys showed that the prepatent period varied from 35 to 98 days (average, 66.2 days). Except for two monkeys (Nos. 13 and 14) the daily egg production was extremely low. The three periods of egg production reported by Vogel and Minning (1935)[2] were observed in these two animals. While the number of eggs was rather high, most of them were immature (Table 1).

Among the 13 monkeys with *S. japonicum* eggs in the stools, autopsy was

[2] "Nach einer einzelnen Erstinfecktion mit Männchen und Weibchen stieg die Eizahl/1 ccm. zunächst an und nahm dann allmählich ab. Auf eine Periode I der Eiausscheidung, in der die Eier regelmässig durch Zählung erfasst werden konnten, folgte eine Periode II, in der die Eier nur zeitweise in zählbaren Mengen auftraten, aber noch fast regelmässig durch den MSV erfasst werden konnten. In Periode III traten spärliche Miracidien nur noch hin und wieder, oft nach langen Pausen, auf."—Vogel and Minning, 1953.

TABLE 3

Results of stool examinations and autopsy findings of 3 monkeys in the J-F series and 4 in the P-F series

Monkey No.	Pre-patent period (Days)	Egg count period: Begin-End (Days after infection)	Maximal daily egg production	Average daily egg production in positive stools			Termination (Days)	Worm finding at autopsy			
				Immature	Mature	Degenerate		Portal vein and its branches		Mesenteric veins	
								M	F	M	F
J-F Ser.											
21	41	41–118	738	23.8	109.8	16.5	118, died	112	112	43	47
22	53	22–303	35	1.6	3.3	0.9	340, living	—	—	—	—
23	41	41–137	296	25.0	23.2	12.1	153, died	21	21	34	32
P-F Ser.											
17	—	—	—	—	—	—	19, killed	—	— (1)	—	—
18	38	28–458(2)	772	14.5	44.9	4.0	473, living	—	—	—	—
19	42	42–91 (2)	1,817	30.6	263.6	7.0	94, died	88	18	23	13
20	51	51–70 (3)	366	—	—	—	70, died	85	25	18	10

(1) Monkey No. 17 was killed by accident on the 19th day of infection. Many immature worms were found in the portal vein and its branches and in mesenteric veins but the number of worms was not counted.

(2) In Monkey No. 18, egg-stage differentiation counts were begun on the 63rd day of the infection and in monkey No. 19, on the 81st day.

(3) In monkey No. 20, egg-stage differentiation counts were not made.

performed only on 6 (Nos. 5, 6, 8, 14, 16 and 26); the other 7 were not sacrificed at the termination of the experiment. Except for No. 14 which showed a high number of worms during autopsy, either no worms (Nos. 5, 6, 16 and 26) or only a few worms (No. 8) could be found. In No. 6, although no worms were found, *S. japonicum* eggs were revealed in the liver by KOH digestion. As the number of days without *S. japonicum* eggs in the stools before autopsy was 205, 120, 134 and 189 for monkeys 5, 6, 16 and 26 respectively, worms might have disappeared in these periods before autopsy.

F-J Series. Eight Formosan monkeys were exposed to the cercariae of the Japanese strain—Nos. 9, 10 and 36 were adults; No. 32 was a young adult; and Nos. 31, 33, 34 and 35 were young. Results of stool examinations and autopsy findings are given in Table 2. The prepatent period varied from 36 to 39 days (average, 37 days). In all cases, the egg production period I of Vogel and Minning was present[3]; maximal daily egg production was high and the number of mature eggs was definitely higher than that of immature ones, especially in period I. The number of worms found at autopsy was also high.

F-P Series. Two adult Formosan monkeys were exposed to the cercariae of the Philippine strain. The results of stool examination and autopsy findings are also given in Table 2. Although daily egg counts were not made, the prepatent

[3] While Nos. 9, 10, 31, 34 and 35 died in the egg production period I, Nos. 32 and 36 lived long enough to show periods I and II and No. 33, all 3 periods.

period was short, 34 to 35 days. Three periods were present and the maximal daily output was high. The proportion of mature eggs was not as high as in the previous series but this is probably due to the fact that most of the counts were made during periods II and III. The maximal egg production figures indicate that the original infections in these two animals must have been rather similar. When No. 11 died and was examined 148 days after exposure, a large number of worms were found. In the other animal, the stools had been negative for 65 days when it was killed 531 days after exposure. Only two worms were found. Apparently this animal had almost recovered from the infection.

J-F Series. Three Japanese monkeys were infected in this series. Monkey No. 21 was a young adult; No. 22, an adult; and No. 23, old. The results of stool examinations and autopsy findings are shown in Table 3. The prepatent period was relatively short, 41 to 53 days (average, 45 days). All 3 monkeys showed egg production periods I, II and III and the maximal daily egg production was high except for No. 22 which was comparatively low. In the two younger animals (Nos. 21 and 22) the number of mature eggs was greater than the immature. The number of worms found at autopsy was high in monkeys Nos. 21 and 23 (No. 22 was not sacrified).

P-F Series. Four young adult Philippine monkeys were used in this series. Results of stool examination and autopsy findings are given in Table 3. Monkey

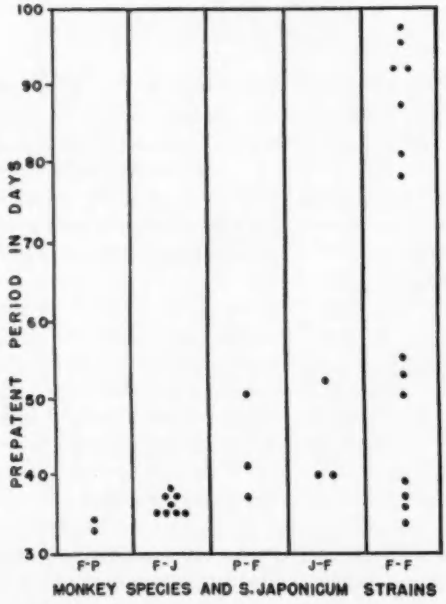

FIG. 1. Prepatent periods of *S. japonicum* in monkeys, each black dot representing one monkey.

No. 17 died accidentally in the prepatent period but many immature worms in the portal vein and mesenteric veins showed that the animal was definitely infected. The prepatent period of the other 3 monkeys varied from 38 to 51 days (average, 43.7 days). Monkeys numbers 19 and 20 died in egg-production period I whereas No. 18 lived long enough to complete periods I, II and III. The maximal number of eggs produced daily was high and the number of mature eggs was much greater than that of the immature eggs. The two animals autopsied during period I of egg production had large numbers of worms.

DISCUSSION AND CONCLUSIONS

While the data are incomplete in some instances and the number of animals used in some of the experiments is small, it is believed that certain conclusions may be drawn. In considering whether the various combinations that were tried were compatible, the length of the prepatent period, number of eggs produced, immature-mature egg ratios, and the number of adult worms at autopsy, appear to be pertinent.

A summary of the data on the prepatent periods in the five series of experiments is shown in Fig. 1. It will be noted that in the F-P, F-J, P-F and J-F series the prepatent period does not extend beyond 53 days. On the other hand, 8 of the 13 monkeys in the F-F series had longer prepatent periods. It is believed that the growth of the worms was retarded in these animals.

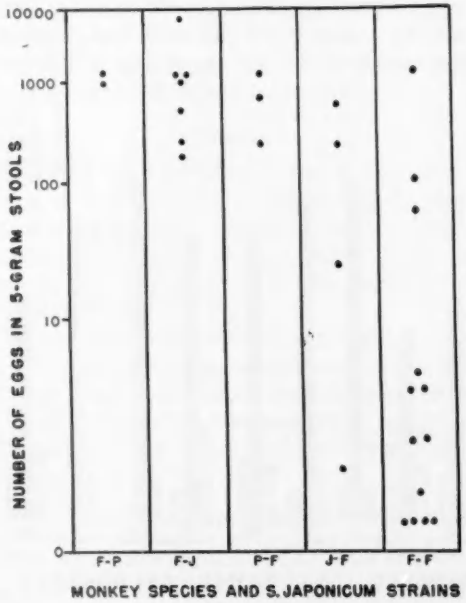

FIG. 2. Maximal daily counts of *S. japonicum* eggs in the stools of infected monkeys, each black dot representing one monkey (semi-logarithmic scale).

Data on mean daily egg production were incomplete in certain experiments so the maximum observed has been used in the comparison of the five experimental groups. The average maximum daily counts for the various series were as follows: F-P, 1309; F-J, 1945; P-F, 985; J-F, 356; and F-F, 161. A summary of the data on individual animals is shown in Fig. 2. The pattern in this graph is essentially the reverse of that seen in Fig. 1. It will be noted that only two of the 13 animals in the F-F group produced as many eggs as those in the other groups.

In considering the data on the relationship between the number of immature, mature and degenerate eggs found in the stools, it was felt that only those observed during period I should be used for comparison. A summary of the data from 14 of the animals examined during this period is presented in Fig. 3. Unfortunately only one animal could be used in this comparison from the F-P series. In this animal 35.9 per cent of the eggs were immature, 56.4 per cent mature and 7.7 per cent degenerate. The average proportions found in six animals in the F-J series was 13.6 immature, 70.6 mature and 15.8 per cent degenerate. In two from the P-F series the average proportions were 16.6, 79.2 and 4.3, respectively. In the three animals from the F-F series the average proportions were 70.0, 19.8 and 10.3. It will be noted that the number of degenerate eggs is relatively constant, but there are marked differences in the immature-mature egg ratios.

The number of worms found in the animals at autopsy also gave some indication of the compatibility between the species of the monkeys and the *S. japonicum* strains. For this comparison only those animals examined 50 to 200 days after exposure were used. One animal in the F-P series had 174 worms. The range in six of the F-J series was 89 to 201, with an average of 133 worms per monkey.

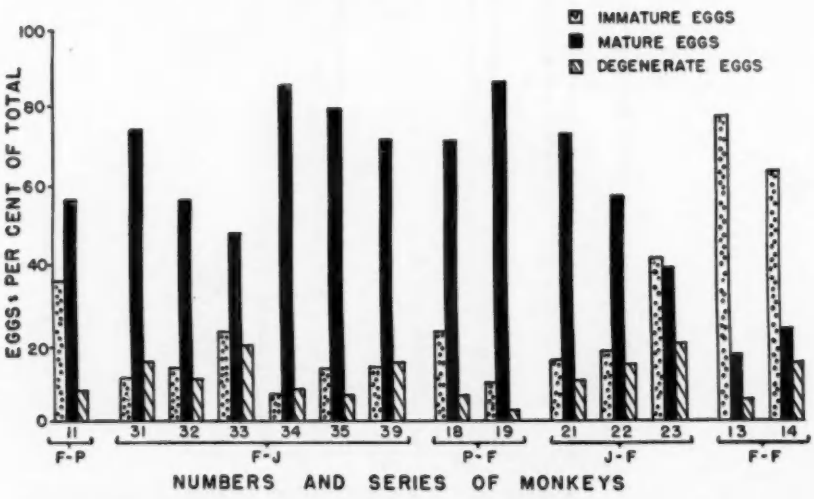

FIG. 3. Proportions of totals of immature, mature and degenerate *S. japonicum* eggs in the stools of monkeys which were observed during period I.

The average in two animals in the P-F series was 140. In two from the J-F series the average was 211. In seven animals from the F-F series one had 300 worms, one had 10, two had only a few and three were negative.

It is apparent that within the groups of animals there was considerable variation. The best example of this is in the F-F series. In monkey No. 14 the prepatent period was short, the number of eggs produced was high and the total worms recovered at autopsy was also high. It would appear that this animal was highly susceptible to the Formosan strain of the parasite. However, it was the presence of large numbers of immature eggs in the feces of this animal that cast doubt on its being an ideal host. The data from the other animals in this group indicate that the average Formosan monkey is not a good host for the local strain of *S. japonicum*.

The above comparison of the prepatent periods, the maximal daily egg output, immature-mature egg ratios, and the number of worms found at autopsy give added evidence that the Formosan strain of *S. japonicum* differs from the Japanese and Philippine strains. The Formosan monkey is highly susceptible to the last two strains but is a relatively poor host for the local one. On the other hand, the Philippine and Japanese monkeys make reasonably good hosts for the Formosan strain of the parasite. This supports our previous suggestion that there were strain differences (Hsü *et al.*, 1954, 1955). While the results obtained in the F-F series may indicate that most human volunteers might not develop full-blown cases of schistosomiasis, the results obtained in the exposure of Philippine and Japanese monkeys to the cercariae of the Formosan strain do not support this idea. The final proof of the inability for the Formosan strain to infect man can be obtained only by exposing human volunteers.

SUMMARY

Thirty-six macaques (29 from Formosa, 3 from Japan and 4 from the Philippines) were exposed to *S. japonicum* cercariae of three geographical strains (Formosan, Japanese and Philippine). The degree of susceptibility of the monkey species to the strains of the parasite was ascertained by the length of the prepatent period, the number of eggs produced, the immature-mature egg ratio, and the number of adult worms at autopsy.

The results of the experiments showed that the Formosan monkey was highly susceptible to the Japanese and Philippine strains but was a relatively poor host for the Formosan strain. On the other hand, the Philippine and Japanese monkeys made reasonably good hosts for the Formosan strain of the parasite. This indicates that the Formosan strain of *S. japonicum* differs from the Japanese and Philippine strains.

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SCHISTOSOMIASIS IN SAUDI ARABIA

Treatment with Lucanthone Hydrochloride (Nilodin) and with Sodium Antimonyl Gluconate (Triostam)

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Data regarding the presence of endemic schistosomiasis in Saudi Arabia, both by *Schistosoma mansoni* and by *S. haematobium*, have been reported in a previous paper (Tarizzo, 1954). As mentioned therein therapeutic trials were carried out with lucanthone hydrochloride (Nilodin, Burroughs Wellcome & Co.); later on trivalent sodium antimonyl gluconate (Triostam, Burroughs Wellcome & Co.) was also used for the treatment of the disease. It is believed worthwhile to report the results obtained not only as these are the first records from this country, but also because the interference of reinfections in the evaluation of the results was excluded. Their significance has been limited by the difficulties encountered in obtaining a regular follow up of the patients.

MATERIALS AND METHODS

Lucanthone hydrochloride and trivalent sodium antimonyl gluconate were administered to two series of unselected and consecutive cases, series I (lucanthone hydrochloride) including 24 cases of *S. mansoni* and 4 cases of *S. haematobium* infections and series II (sodium antimonyl gluconate) 41 and 5 cases respectively. Nineteen cases of *S. mansoni* infections were clinically asymptomatic and had been detected on routine examination. All cases of *S. haematobium* infections had gross hematuria. With the exception of a few cases treated during hospitalization, all other cases were treated as outpatients and continued their normal activities, except when they developed toxic reactions.

Lucanthone hydrochloride was administered by mouth in plain tablets in 3 daily doses, over a period of 4 to 10 days. Sodium antimonyl gluconate was given intravenously—dissolved in distilled water—in 4 to 6 doses on consecutive or, later on, on alternate days.

The success of the treatment was established by the disappearance of the eggs from the excreta for at least 3 consecutive examinations. Patients were requested to submit specimens once a week: feces were examined by direct examination and after concentration, and urine after sedimentation and centrifugation. In addition, hatching tests were carried out on specimens negative on microscopic examination.

None of the patients left Dhahran during the period of treatment and follow up. As the disease is not present in the immediate vicinity the reappearance of the eggs in the excreta was, therefore, considered as a failure of the treatment.

[1] The author wishes to thank Dr. R. C. Page, Medical Director, Arabian American Oil Company, for permission to publish this paper.

TOXIC REACTIONS

Of the 24 cases with *S. mansoni* infections treated with lucanthone, 10 developed symptoms to be considered as toxic reactions. The incidence was the following: nausea or vomiting in 7 cases, abdominal and epigastric pain in 5, yellow discoloration of the skin in 2, diarrhea in 1. In 2 cases the treatment had to be discontinued due to the severity of the reactions. Of the 41 cases treated with sodium antimonyl gluconate, 13 showed untoward effects during or immediately after the treatment: 7 patients had nausea or vomiting, 5 developed a skin rash, 2 pruritus, 2 cough, and 1 low grade fever. In 2 cases the treatment had to be discontinued because of the reactions. The toxic symptoms could—in part, at least—be controlled with the administration of antiallergic drugs and they all disappeared a few days after the discontinuation of the drug without evidence of permanent damage. No reactions to the treatment were observed in any of the cases with *S. haematobium* infections.

RESULTS

Regular follow up after treatment for a period of at least 4 weeks could be obtained only in 16 cases of series I (12 with *S. mansoni* and 4 with *S. haematobium*) and in 22 of series II (18 and 4 respectively); only these, therefore, are considered in the discussion of the results as no significant conclusions could be reached in the other cases. Details regarding these patients, doses, and results are included in Table 1. All these patients were males: this and the limited age range are explained by the fact that most of the cases were Company employees.

S. mansoni infections. With lucanthone hydrochloride a cure was obtained in 6 out of 12 cases with dosages from 55 to 75 mg./kg. body weight. With sodium antimonyl gluconate a cure was obtained in 12 out of 18 cases with dosages varying from 13 to 21.6 mg./kg. body weight. The period of follow up was from 4 to 39 weeks. Only in the cases observed for several months after treatment could a clinical improvement be detected, with gradual disappearance of the complaints—if present—and subjective feeling of well being. In the few cases in which anemia was present, this responded to the treatment without the administration of any antianemic drug. The eosinophilia almost always increased during treatment; this was a transient phenomenon lasting not more than a few weeks. Liver function tests showed a more retarded response and complete return to normal values was not observed in any of the cases which had shown marked liver damage before treatment.

S. haematobium infections. With lucanthone hydrochloride a cure was obtained only in the patient who received a total of 110 mg./kg. body weight; with sodium antimonyl gluconate a cure was obtained in all 4 cases treated with a dosage of 20 to 24 mg./kg. body weight. They were followed up for 4 to 29 weeks. The hematuria and dysuria disappeared and the urine remained completely normal on examination. The increase in eosinophilia was observed also in these cases, but was less pronounced and the clinical response to the treatment was much more rapid.

TABLE 1

Cases of schistosomiasis treated with lucanthone hydrochloride (Nilodin) and with sodium antimonyl gluconate (Triostam) in Dhahran, Saudi Arabia

Case	Age	Origin	Weight	Total dosage	Days of treatment	Follow up	Result
Series I: Lucanthone hydrochloride (Nilodin)							
			kg.	*mg./kg.*		*weeks*	
S. mansoni							
1. A. A.....	18	Bisha	45	65	5	9	Cure
2. A. M.....	19	Yemen	46	65	5	12	Failure
3. A. I.....	21	Mecca	52	57	6	8	Cure
4. A. M.....	22	Bisha	46	65	5	4	Failure
5. A. F.....	25	Riyadh	54	55	5	4	Failure
6. G. A.....	30	Yemen	54	66	6	9	Cure
7. M. A.....	12	Riyadh	26	58	5	11	Failure
8. M. A.....	15	Ghamid	40	75	5	5	Cure
9. M. M.....	20	Sudan	61	60	5	4	Failure
10. M. N.....	25	Riyadh	51	60	5	25	Cure
11. M. M.....	20	Abha	54	55	6	4	Failure
12. H. A.....	20	Majma'a	42	57	4	22	Cure
S. haematobium							
13. R. M.....	21	Hail	61	60	5	5	Failure
14. O. A.....	30	Hadhramaut	54	110	10	29	Cure
15. S. A.....	20	W. Shahran	53	57	5	15	Failure
16. A. M.....	20	Hadhramaut	51	60	5	4	Failure
Series II: Sodium antimonyl gluconate (Triostam)							
S. mansoni							
1. H. T.....	38	Majma'a	55	19	6	13	Cure
2. M. N.....	23	Majma'a	50	21.6	6	9	Cure
3. A. M.....	30	Eritrea	68	16.5	5	31	Cure
4. A. M.....	30	Aden	48	17	6	10	Failure
5. A. S.....	22	Nejd	55	13	4	39	Cure
6. N. R.....	24	Aden	68	19.8	6	25	Cure
7. J. G.....	22	Riyadh	45	20	6	31	Cure
8. M. M.....	14	Hail	26	20	6	14	Failure
9. A. I.....	24	Nejd	45	20	6	13	Failure
10. M. M.....	12	Riyadh	30	18	12	17	Cure
11. M. M.....	22	Al Jauf	70	19.2	12	6	Failure
12. M. A.....	30	Aden	47	17.2	12	7	Cure
13. S. A.....	22	Hejaz	55	19.6	12	5	Cure
14. H. T.....	35	Mecca	52	20	12	14	Cure
15. A. H.....	20	Ghamid	54	20	12	5	Cure
16. K. N.....	24	Aden	61	17.7	12	5	Cure
17. S. A.....	26	Riyadh	56	20	12	9	Failure
18. M. A.....	31	Eritrea	61	17.7	12	8	Failure
S. haematobium							
19. M. A.....	25	Yemen	54	20	6	9	Cure
20. A. M.....	17	Hejaz	54	20	12	5	Cure
21. H. A.....	36	Hejaz	45	24	12	5	Cure
22. B. A.....	22	Hadhramaut	60	20	12	5	Cure

DISCUSSION AND CONCLUSION

In the available literature the results obtained with lucanthone hydrochloride against *S. mansoni* have been somewhat discordant with reported cure rates ranging from 9 per cent (Fain and Lagrange, 1952) to 90 per cent (Janssen, 1951), whereas more constant results have been obtained against *S. haematobium* with cure rates averaging 60 to 90 per cent (Blair *et al.*, 1941; Alves, 1949, 1950; Watson *et al.*, 1951, 1952) and with a markedly lower incidence of toxic reactions. The effective dosage appears to be in the order of 100 mg./kg. body weight or higher, but this dosage is seldom tolerated by patients with *S. mansoni* infections, who also seem to require a more intensive treatment.

Only a few reports are available on the clinical use of sodium antimonyl gluconate. It has been used in Egypt (Erfan and Talaat, 1950), in Iraq (Watson and Pringle, 1950), and on the Gold Coast (Messent, 1954) against *S. haematobium*, and in Brazil against *S. mansoni* (Da Silva and Dias, 1951). The cure rates reported have been of 73, 100, 56, and 72 per cent respectively, with dosages of 18 to 36 mg./kg. body weight; toxic reactions have been rarely observed.

The results reported in this paper, although in a limited number of cases, are in agreement with those obtained elsewhere. As with other drugs, infections with *S. haematobium* are more easily eradicated and the treatment causes less discomfort to the patient. Whether this is to be related to the different localization of the parasites or to an intrinsic difference of the two species in the response to the various drugs, is yet to be determined. Impossibility of obtaining more detailed information in many of the cases did not permit any conclusion regarding a possible relationship between duration of illness or intensity of infection and response to the treatment.

Lucanthone hydrochloride has not proved to be satisfactory for the treatment of *S. mansoni* infections as the dosage tolerated by the patients gives only a limited cure rate. The drug is undoubtedly effective against *S. haematobium*, but conclusions cannot be presented due to the limited experience.

Sodium antimonyl gluconate presents definite advantages, when compared with all other available drugs, especially against *S. mansoni*. It is relatively well tolerated and satisfactory results can be obtained with comparatively low dosages and in a relatively short period of time.

SUMMARY

In *Schistosoma mansoni* infections in Saudi Arabia a cure was obtained in 6 out of 12 cases with lucanthone hydrochloride (Nilodin) in dosages of 55 to 75 mg./kg. body weight, and in 12 out of 18 cases with sodium antimonyl gluconate (Triostam) in dosages of 13 to 21.6 mg./kg. body weight. In *S. haematobium* infections, one case was cured with 110 mg./kg. of lucanthone hydrochloride and 4 out of 4 cases were cured with 20 to 24 mg./kg. of sodium antimonyl gluconate.

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FATAL HEMATEMESIS IN *SCHISTOSOMA MANSONI* INFECTION

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Schistosoma mansoni adults are usually located in the inferior mesenteric venules. They may also be found in the tributaries of the superior mesenteric vein, in the portal vein radicles within the liver, in the hemorrhoidal venules, and occasionally in the pulmonary arterioles. On the other hand, *Schistosoma japonicum* adults are found chiefly in the superior mesenteric venules, but may also be located in the small tributaries of the inferior mesenteric vein and in the intra-hepatic portal veins. *Schistosoma haematobium* adults are observed primarily in the pelvic plexuses, especially the vesical venules, but may be seen in the mesenteric venules or rarely in the branches of the portal vein within the liver and the pulmonary arterioles.

Worms have been reported occasionally in ectopic locations. El Gazayerli (1939) found a male *S. haematobium* in the circumflex branch of the left coronary artery, and Badir (1946) found a pair of worms in a branch of the superior ophthalmic vein when he removed a tumor from the conjunctiva of an Egyptian boy. Japanese investigators (Fujinami *et al.*, 1913; Yokogawa and Morishita, 1931) have reported *S. japonicum* adults in experimentally infected animals in various ectopic locations, including the middle cerebral vein of a monkey, esophageal vein of a horse, and right heart, vena cava, gastric vein, splenic vein, and right renal vein of a pig. In all the reported cases the ectopic worms were within vessels with no surrounding inflammatory reaction, indicating that they were freely circulating and not producing vascular obstruction. In the following case report *S. mansoni* worms were found in the gastro-esophageal veins as well as in the usual locations.

CASE REPORT

A 28-year-old male Puerto Rican was admitted to Jackson Memorial Hospital at Miami, Florida, at 6:50 A.M. complaining chiefly of pain in the abdomen. About four hours prior to admission he was awakened by epigastric pain, approximately thirty minutes after which he vomited "more than three quarts" of black tarry material and some bright red blood. The patient had never been sick and enjoyed excellent health until about five months prior to admission, when he had a bout of gnawing epigastric pain lasting approximately two hours and relieved by milk of magnesia. About one month before admission he passed several black stools, and was not taking iron or any similar medication. The family history was not remarkable except that one brother died of "ruptured stomach". Review of systems was non-contributory.

Physical examination on admission revealed a well-developed, well-nourished white male in no acute distress. The pulse rate was 88 per minute and regular.

The blood pressure was 94/52 mm. Hg and respiration was regular at 20 per minute. With the exception of slight left lower quadrant tenderness and a palpable spleen one to two finger breadths below the costal margin, the remainder of the physical examination was essentially negative.

At the time of admission the patient vomited about a pint of blood. An immediate hematocrit was forty per cent. An upper gastro-intestinal series suggested duodenal ulcer. During the next 24 hours after admission he had six more episodes of hematemesis and the hematocrit fell to 26 per cent despite transfusion of eight pints of whole blood. Twelve hours later after seven more pints of whole blood had been transfused, the hematocrit was 30 per cent. At 8:00 P.M. on the second day after admission the patient was losing more blood than he was receiving, even though two transfusions were being given simultaneously. During this period three pints of whole blood were given in less than thirty minutes. At this time the patient's blood pressure was 110/60 mm. Hg and the pulse was 100 per minute.

The patient was scheduled for emergency surgery at 9:50 P.M. During the induction of anesthesia, he vomited a large amount of blood and aspirated part of it, necessitating bronchoscopy. Following this procedure the abdomen was opened and explored. The stomach, small intestine and colon were found to be dilated and full of blood. A gastrostomy was performed close to the duodenum, and after duodenal bleeding was ruled out, the stomach was opened to its midportion. During this procedure the gastric mucosa was bleeding so profusely that continuous suction could not keep it free of blood. No gastric ulcer could be found. Esophageal varices were ruled out after inflation of a Miller-Abbott double balloon failed to stop the bleeding. Finally on the lesser curvature close to the cardia a large bleeding vessel was located and ligated. However, the entire gastric mucosa, especially that of the fundus, continued to ooze and bleed. Because of the very poor condition of the patient, who was in deep shock, gastrectomy was not done.

The patient died three hours after the operation. A total of 29 pints of whole blood had been given since admission.

AUTOPSY AND MICROSCOPIC FINDINGS

The autopsy was restricted to the abdomen. External examination revealed a well-nourished, well-developed white male with a recent vertical incision extending from a point two centimeters to the left of the xyphoid process to a point 1.5 centimeters to the left of the umbilicus. The sutured incision in the abdominal wall was intact and there was minimal hemorrhage in the surrounding tissue. The peritoneal cavity contained about 2,500 cc. of blood. The abdominal organs were in the proper anatomical relationship.

The liver was only slightly decreased in size, weighing 1,410 grams. The capsule was irregularly thickened by multiple, small, patchy, greyish-white areas averaging one to three centimeters in diameter. The consistency was moderately increased and the liver edges were slightly blunted. On section the peri-portal fields were very prominent with greyish-white thickening, producing a cuffing

FIG. 1. Liver with fibrosis prominent about veins

effect around the portal veins (Fig. 1). The intervening parenchyma was dull brown. Microscopically, many of the peri-portal fields showed extensive fibrosis with infiltrations of lymphocytes, eosinophils and plasma cells (Fig. 2). Scattered here and there through the dense connective tissue of these areas were numerous eggs. Most of these were distorted; however, the characteristic prominent lateral spine of *S. mansoni* could be demonstrated in many. Some were surrounded by a fringe or halo of amorphous eosinophilic material (Fig. 3), others appearing merely as clear refractile shells with large multinucleated giant cells and associated epithelioid cells. The Kupffer cells contained large amounts of dark brown non-refractile granular pigment which failed to stain with Prussian blue. No worms were seen in the intra-hepatic portal veins. The gall bladder revealed no abnormalities grossly or microscopically.

The spleen was somewhat pale and enlarged, weighing 290 grams. The capsule was wrinkled but showed no thickening or adhesions. On section the parenchyma appeared pale reddish-brown and the trabecular markings were somewhat promi-

FIG. 2. Section of liver showing large area of fibrosis with scattered eggs and inflammatory cells.

nent. Microscopically, focally scattered small hemorrhages were seen. The trabeculae were slightly increased in thickness by fibrous connective tissue and the follicles were inconspicuous. Dark brown non-refractile granular pigment, similar to that seen in the Kupffer cells of the liver, was observed in the reticular cells.

The adrenal glands and genito-urinary organs were grossly normal and microscopically presented congestion.

Grossly, no varices were seen in the inferior portion of the esophagus. The 10-centimeter-sutured gastrostomy was noted to extend from the pyloric region toward the fundus. There was extensive hemorrhage into the gastric wall in all layers of this incision. Near the lesser curvature of the stomach, 1.5 centimeters from the cardio-esophageal junction, there was a single suture with extensive surrounding hemorrhage in the wall of the stomach. The stomach was filled with blood and the rugae were flattened. The fundic mucosa revealed numerous

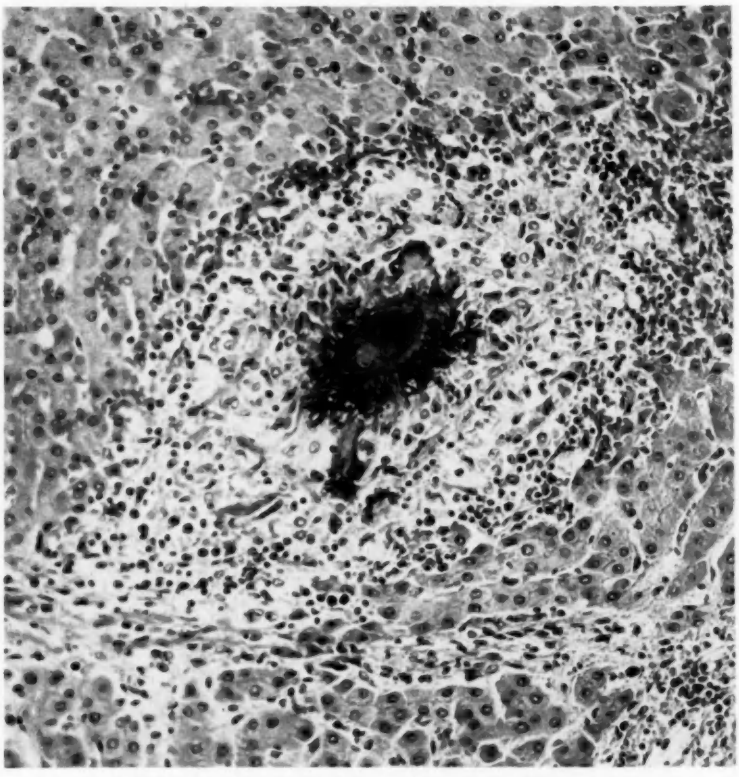

FIG. 3. Hepatic pseudotubercle with central egg and halo

petechiae and ecchymoses. In the mucosa of this region, overlying the previously described single suture, there was a soft hemorrhagic puckered area with no visible open vessel or ulceration. The small and large intestines were filled with dark red blood. The small intestine was grossly normal; however, the wall of the large intestine appeared slightly thickened. The mucosa was intact with no gross evidence of bleeding points, ulceration or inflammation.

Microscopically, the veins in the submucosa and serosa of the esophagus and adjacent stomach in the vicinity of the cardio-esophageal junction were dilated and many contained one or more male and female, single and copulating worms (Fig. 4). There was no inflammatory reaction in or about the veins containing the worms. Multiple sections disclosed only a few eggs in the fundic gastric mucosa located within one local 7 millimeter area. There was a surrounding inflammatory reaction consisting almost entirely of eosinophils. Apart from the eggs there was a light infiltration of eosinophils in the gastric mucosa and a few focal collections of eosinophils without associated eggs. Scattered and focal con-

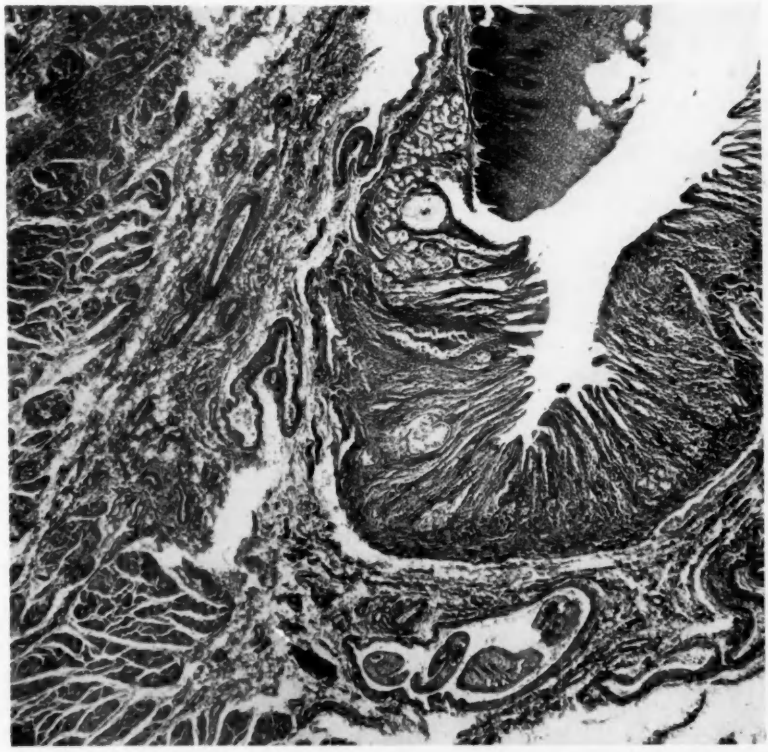

FIG. 4. Worms in gastric submucosal vein near cardio-esophageal junction

centrations of lymphocytes were also noted. No pseudotubercles were present. Many sections showed extensive hemorrhage in the gastric submucosa, muscularis, and serosa with involvement of the lower esophagus. The mucosa of the small intestine and colon contained numerous eosinophils as well as lymphocytes. Within the mucosa and submucosa of the jejunum, ileum, and large intestine there were numerous eggs and pseudotubercles. Neither were present in the duodenum. Multiple sections revealed only two copulating pairs of worms in the submucosal venules of the jejunum. Only one worm was seen in a submucosal venule of the colon. No other worms were found. Grossly and microscopically the pancreas disclosed no pathologic changes.

DISCUSSION

It is well known that adverse conditions in the usual locations inhabited by schistosomes may stimulate the worms to migrate elsewhere. After insufficient therapy most of the worms may be found in the intra-hepatic portal veins at

autopsy. Surgical procedures, such as biopsy, may also cause migration to ectopic locations (Faust, 1948).

With the development of cirrhosis and consequent blockage of the portal circulation, accessory venous channels are dilated to provide collateral circulation to offset the intra-hepatic obstruction. In these cases much of the venous flow, coming from the mesenteric veins (and hence venules usually inhabited by the adult worms), by-passes the obstructed intra-hepatic portal veins via the gastro-esophageal veins. The latter, having undergone considerable compensatory dilatation, receive and convey much of the mesenteric venous blood which would ordinarily have flowed through the liver.

In this case the patient was in shock for a rather long period of time. This undoubtedly led to changed environmental conditions in the usual habitat of the worms and may have provoked migration, which may also have been influenced by the surgical procedure. The tremendous amount of blood given this patient (and subsequently lost through the ruptured varices) undoubtedly produced a marked flushing effect in the mesenteric venous flow. Thus, any one or combination of the above hemodynamic and/or traumatic factors may have caused the movement of the worms to their ectopic location.

In spite of the large number of worms found in the veins at the cardioesophageal junction careful search of numerous sections of this region revealed only a few eggs. There was a surrounding infiltration of eosinophils about the eggs and no pseudotubercles or fibrosis were noted, indicating relatively recent development. As opposed to this, numerous eggs, pseudotubercles, fibrotic changes, and a few worms were observed in the intestine.

Because of the location of almost all of the eggs and all of the pseudo-tubercles in the bowel, and since only a few eggs together with a surrounding infiltration of eosinophils and no pseudotubercles or fibrosis were found in the gastric wall in association with the numerous adult worms seen there, it is reasonable to suppose that the ectopic position of the worms in the gastro-esophageal veins was due to a terminal mass migration occurring either just before or during the period of active gastric bleeding. It is not possible, however, to prove whether the migration occurred before or after the rupture of the gastric veins. If this migration took place prior to the bleeding, the blockage of numerous venous channels by the worms themselves may have caused an additional rise in pressure in vessels already under stress from portal hypertension. In addition, eggs laid by these worms might promote rupture of the veins by means of lytic substances, the piercing action of the spine, and other effects. Thus these factors may have contributed directly to the rupture of the gastric veins. Of course, it is well known that in cirrhosis varices may rupture as the result of the portal hypertension alone.

SUMMARY

A fatal case of bleeding gastric varices, resulting from cirrhosis of the liver produced by eggs of *Schistosoma mansoni* and associated with worms in the varices, has been presented. Factors that might account for the unusual location

of the worms and the possible significance have been discussed. It is suggested that a careful post-mortem search of the cardio-esophageal veins in similar cases of persons who had lived in endemic foci of schistosomiasis might lead to the discovery of worms in this ectopic position.

ACKNOWLEDGEMENT

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ORAL AND INTRAPERITONEAL TREATMENT OF *ACANTHOCHEILONEMA (DIPETALONEMA) PERSTANS* WITH DIETHYLCARBAMAZINE

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It is a matter of experience that *Acanthocheilonema perstans* as a rule causes few or no symptoms of illness. Numerous authors however report cases which showed definite symptoms, e.g., Teichler, 1938; Molser, 1939; Garratt, 1945; Heckenroth, 1950; and others. Bourguignon, 1937, even mentions a fatal case, caused by the concentration of microfilariae of the perstans type in the liver.

The first therapeutic attempt is reported in 1939 when Molser described the use of intravenous injections with a one per cent solution of methylene blue. Later other experiments with various treatments were reported. Temkin (1945) published a list of over 100 different chemical compounds which up to then had been tried as filaricides, but which had not produced convincing results. Since 1947, 1-diethyl-carbamyl-4-methylpiperazin (diethylcarbamazine) has prevailed as a successful filaricide in the treatment of *Wuchereria bancrofti*, *Wuchereria malaya*, *Onchocerca volvulus* and *Loa loa*.

There are numerous publications on the results of treatment of *A. perstans* with diethylcarbamazine: Murgatroyd and Woodruff (1949); Shookoff and Dwork (1949); Wanson (1949); German *et al.* (1950); Heckenroth *et al.* (1950); Almeida (1952); McGregor *et al.* (1952). The majority of the above mentioned authors come to the conclusion that diethylcarbamazine is not effective in *A. perstans* or causes only a temporary disappearance of microfilaria, although a few report success.

In my capacity as Medical Officer of a construction project at the source of the river Nile below Lake Victoria, (Jinja, Uganda Protectorate) I had an opportunity to observe a small number of patients suffering from *A. perstans* and to undertake therapeutic experiments over a long period. Owing to a rapid turnover of labor, the medical staff of the company, in the course of three years, had cared for some 16,000 natives from many different tribes of Central Africa, totaling with their families, about 20,000 in all. Among these, 152 cases (0.8%) were diagnosed as *A. perstans* by bloodslides; 39 of these (26%) showed symptoms obviously due to *A. perstans*, and were therefore admitted to the company's Hospital for treatment. Seven patients (4.6%) had very severe symptoms, which, after the elimination of other causes, were attributed to the presence of *A perstans*.

In January, 1945, it was necessary to obtain bloodslides from all the African workers of the company and their families because of the occurrence of a case of sleeping sickness. As a result it was found that out of 2,300 men, 39 (1.7%) suffered from *A. perstans*, and out of 481 women and children there were two cases, or 0.4 per cent. The general symptoms observed were as follows:—

subjective: giddiness, aching limbs, periodic itching, abdominal and pectoral pains.

objective: slight swelling of the spleen and liver, occasional fever attacks followed by urticarial exanthema.

The 7 serious cases developed severe symptoms, including edema of the lower limbs. In two cases massive edema of the scrotum and prepuce were observed. The number of microfilaria in thick drop preparations varied considerably and bore no relation to the seriousness of the symptoms.

Precise information regarding the duration of the illness is usually not to be obtained from the natives, because other diseases, such as malaria, measles, chickenpox, dysenteries, etc., which may have occurred during the same period, make an evaluation of the medical history very dubious. It should be noted that the absence of seasonal changes in the tropics makes chronology very difficult, and conscious and unconscious exaggerations are a typical feature of the primitive psyche.

Diethylcarbamazine was used in 1951 in the above mentioned 36 cases of *A. perstans* which were admitted to our company hospital. The dosage was carefully increased in each case, keeping check on any side effects, until a daily dose of 500 mg. by mouth for ten days was reached. The only side effects which could be observed in all patients was an urticaria-like skin reaction, which has been described also by other authors. These itching skin rashes are doubtless to be considered as allergic reactions to the proteins released by the decomposition of the microfilariae killed by diethylcarbamazine. These reactions can be reduced considerably by simultaneous treatment with antihistamines.

The previously mentioned reports by other authors agree that diethylcarbamazine is by no means without some efficacy against *A. perstans*. This fact was also confirmed in the 36 cases under my own observation. In all of these cases there was a considerable improvement in objective symptoms, such as swelling of liver and spleen, edema, and fever, besides relief of subjective symptoms. After the conclusion of the treatment no microfilariae could be found in the blood of 34 of the 36 cases for a period which varied from a week to three months. No definite relation between this period and the dosage used in treatment could be established. In two cases, in spite of maximum dosage (500 mg. a day for ten days) microfilariae appeared in the blood immediately after conclusion of treatment, though in very small numbers.

Combining the reports of others with my own observations, I reached the following conclusions: in sufficient oral dosage, diethylcarbamazine is effective against the microfilariae of *A. perstans*. It can therefore be assumed that it would be effective against the mature parasites as well, provided it were possible to obtain a sufficient concentration of the drug at their location in the peritoneal connective tissue. Presumably the reaction of the peritoneal tissue leads to the formation of an inflammatory barrier around the parasites which prevents the penetration in effective quantities of the diethylcarbamazine carried by the blood and lymphatic stream.

The success of oral treatment with diethylcarbamazine reported by several

authors may be explained as follows: (a) a short duration of the infection or a constitutional or temporarily reduced sensitivity of the peritoneal tissues might prevent the development of the inflammatory barrier and permit a concentration of diethylcarbamazine sufficient to eradicate the filariae; (b) microfilariae are known to disappear from the blood of both treated and untreated patients for weeks or even for a period of two months, after which they reappear. Unless control slides are taken weekly or at least monthly for more than 4 months conclusions as to permanent cure are unreliable.

These theoretical considerations led to the experimental intraperitoneal application of diethylcarbamazine in two cases of *A. perstans* infection. Harned *et al.* (1948) have reported an experimental intraperitoneal injection in animals. It was shown that mice, rats, rabbits and dogs can tolerate doses of 100 mg./kg. without difficulty. Daily intraperitoneal injections of 50 mg./kg. in rabbits, and 100 mg./kg. in rats for 5 to 15 days did not lead to any toxic symptoms. Accordingly it was to be expected that similar treatment in careful dosages would not be dangerous to man.

The following method was chosen: a small laparotomy was performed on the left side under local anesthesia, and a rubber drain introduced under the omentum to the center of the abdominal cavity. The wound was then closed around the drain, allowing the outer end of the tube to protrude some 5 cm. It could be tied with silk or closed with a glass stopper and injections made through it into the abdomen. To prevent secondary infections, 800,000 I.U. of penicillin were given daily throughout the treatment. A 0.5 per cent sterile solution of pure, water-soluble diethylcarbamazine powder was used for injection. So far, two patients have been treated by the method described.

The first patient received intraperitoneal diethylcarbamazine treatment exclusively, in the following dosages: 50 mg. the first day; 100 mg. daily (in divided doses) the next 4 days; and 150 mg. daily, t.i.d., for the next five days, making 10 days in all. These injections caused some pain which lasted about half a minute and was described as quite bearable. The side effects were the same as with oral application of diethylcarbamazine in filariasis: occurrence of itching from the second to the 4th. or 5th. day. The patient was otherwise well. The first bloodslide taken immediately after the conclusion of the treatment showed no microfilariae, but an increase of eosinophils from 30 per cent before the treatment, to 40 per cent after it. However, 2 weeks after the conclusion of the treatment, microfilariae were found again in the blood.

The second patient was under medical observation from the beginning of 1952. Only minor illnesses and an attack of malaria were recorded until July, 1953. At that time he was admitted to hospital with fever, giddiness, pains in the chest, edema of both limbs, and enlarged liver and spleen. The bloodslide showed a distinct increase in eosinophils and the presence of microfilariae of the *perstans* type.

After treatment with 450 mg. of diethylcarbamazine daily by mouth for 10 days, the microfilariae disappeared from his blood for one month. With the reoccurrence of the microfilariae, the previous symptomatology of the patient

returned. After insertion of a drain in the manner described, he was treated as follows: 1st. day: 450 mg. of diethylcarbamazine by mouth (in 3 doses) and 10 cc. of a 0.5 per cent solution of the same drug (50 mg.) intraperitoneally; 2nd. day: 450 mg. of diethylcarbamazine by mouth and 20 cc. of the solution (100 mg.) intraperitoneally; 3rd. to 10th. day: 450 mg. of diethylcarbamazine by mouth and 30 cc. of the solution (150 mg.) intraperitoneally.

In addition during the 10 days, a daily dose of 800,000 I.U. penicillin was given parenterally, and three times daily, 1 tablet of "Antistine". Except for transient pain during the injections of the solution into the abdomen and the usual itching from the second to the fifth day, there were no side effects.

Blood specimens were examined at weekly intervals until the end of March, 1954. No microfilaria could be found, and a decrease of the eosinophilia occurred. The patient felt fit and went to work again. Negative blood tests for the next 7 months as well as the general condition of the patient indicated that he was in fact cured of the attack of *A. perstans*.

DISCUSSION

Oral application of diethylcarbamazine, even in large doses up to 450 mg. per day for 10 days is evidently insufficient to cure an *A. perstans* infection. Even a repetition of the treatment does not achieve a cure, but only a transitory improvement. Intraperitoneal injection of diethylcarbamazine however, in 0.5 per cent sterile solution (10 ccm. 3 times daily), is more promising and can be given without causing severe side effects. By changing the concentration of the diethylcarbamazine solution it is likely that a painless intraperitoneal application is possible. Probably the daily doses can be increased without danger and can thus be made more effective. It can also be expected that the technique will be improved and simplified. A combination of oral and intraperitoneal treatment was successful in one case, and while no general conclusions can be drawn, this success can be considered encouraging for further experiments. Completion of the Owen Falls Hydro-Electric Scheme in the near future, with reduction of the labor force and the consequent closing of the Company's hospital will make further experiments impossible in this area.

SUMMARY

Of 152 cases of *Acanthocheilonema perstans* infections in a Uganda labor force, 39 were hospitalized because of symptoms which in 7 cases were unusually severe.

Treatment with diethylcarbamazine by mouth even in large doses failed to achieve a permanent cure, and injection of the drug into the abdominal cavity was tried in 2 cases. The first relapsed but the other, which received a combined oral and intraperitoneal course of treatment was probably cured, since the blood remained negative for microfilaria for seven months, when examination was discontinued.

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EFFECT OF TOPICALLY APPLIED DDT ON DEVELOPMENT OF *PLASMODIUM VIVAX* AND *P. FALCIPARUM* IN *ANOPHELES QUADRIMACULATUS*

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During the course of residual spray treatment of buildings in an area of malaria endemicity, it is highly probable that some mosquitoes, freshly fed upon an infectious inhabitant, will receive a dose of the insecticide sufficiently meager to permit recovery of the insect. The question has arisen as to whether such a sub-lethal dose of an insecticide will effectively inhibit the growth of, or actually destroy, the parasite in the mosquito (Gabaldon, 1953).

In order to gather data on this subject, the development of the *Plasmodium* was observed in mosquitoes which, having been allowed to bite a paretic patient undergoing malaria therapy, were topically treated with a sub-lethal dose of DDT.

MATERIALS AND METHODS

The mosquitoes used in these experiments were the Q-1 strain of *Anopheles quadrimaculatus*. This colony has been in continuous existence for twelve years at the Columbia, South Carolina, Laboratory of Tropical Diseases. The plasmodia used were *Plasmodium vivax* (St. Elizabeth strain) in five experiments, and *P. falciparum* (McLendon strain) in six experiments.

The mosquitoes were from five to seven days old when given the infective blood-meal. They had been kept in small (one cubic foot or less) plastic-screen cages located in an insectary at 76 ± 2°F. For food, plastic sponges soaked with a five per cent solution of syrup (Dark Karo) were placed upon the cages each evening. The night before the blood-meal was offered the mosquitoes were either starved or given water only.

At varying times (from 4 to 12 hours) before the blood-meal, the mosquitoes were confined, five each, in the larger of the two sizes of plastic jars described by Young and Burgess (1946). These jars measured two and a half inches high by two inches in diameter. The ends were closed by a piece of either nylon or cotton net held in place by a rubber band. Except for the few minutes when they were anesthetized and being treated, the mosquitoes remained in these jars throughout the entire period of feeding and incubation until they were dissected.

The jars of mosquitoes were transported to the hospital ward in a towel-covered container. There they were allowed to bite a neurosyphilitic patient with an active malaria infection. Blood smears were routinely made at eight o'clock in the morning and at feeding time.

Approximately one hour elapsed between the time the mosquitoes became engorged and the application of the DDT. Two variations in timing were tried: in the *P. vivax* No. 5 experiment, treatment was done at the bedside; in the *P.*

falciparum No. 3-E experiment, the mosquitoes were treated about four hours prior to the blood-meal.

Anesthetization of the mosquitoes with CO_2, as devised by Williams (1946), was accomplished by placing a jar of them in a funnel supported by a rack. The funnel was connected by rubber tubing to a tank of the gas. Between the tank and the funnel the tubing was broken by a water trap through which the gas bubbled. The CO_2 was allowed to bubble briskly through the water but not so fast that drops collected in the tubing.

While the mosquitoes were anesthetized the net cap of the jar was removed and the insects dumped onto a white surface. Nonengorged specimens were killed, the rest treated and replaced in the jars.

Treatment with the DDT solution, after the manner of Ludvik (1953), was done in three steps:

1. A calibrated wire loop was dipped into acetone to a depth of about one-half inch and dried by touching to a clean blotting paper. This insured cleanliness of the loop.

2. The loop was next dipped into the DDT solution, quickly withdrawn, and applied to the integument of the insect.

3. To prevent accumulation of the insecticide the loop was again rinsed in a second jar of clean acetone.

At intervals during the treatments the jars of acetone and DDT, as well as the loops, were rinsed with clean acetone and new solution provided. This furnished added insurance against accumulated deposits of DDT on the equipment. Such things as small differences in the depth to which the loop was dipped, the speed with which the operation was done, the actual spot upon the integument at which the application was made, or even the interference of legs and wings during the process, could influence the amount of the DDT deposited on the insect. Since uniformity was of primary importance great care was used during the process of application and but one operator conducted the treatments for each experiment.

After treatment the mosquitoes were replaced on the insectary shelves for incubation of parasites. The jars were placed in enamel-ware pans and a syrup-saturated cloth was placed over them nightly for sustenance.

In order to determine the oocyst densities, gut dissections were made on the eighth or tenth day depending on survival. Still later, if enough mosquitoes remained alive, salivary gland dissections to determine sporozoite densities were done.

Gut indices and gland indices based on the averages of the plus ratings of infected guts or glands were computed after the formulae of Young *et al.* (1946). Infective indices, which were the theoretical number of oocysts in 100 dissected guts, were computed by multiplying average oocysts per infected gut by the percentage of infection and again by 100.

RESULTS

Effect of treatments on mosquito survival. In order to determine the mortality caused by the treatments, two censuses were taken on each of the *P. vivax*

TABLE 1

Effect of topically applied DDT on the survival of Anopheles quadrimaculatus *after engorgement of malarial blood*

	Group	Per cent survival after treatment		
		1st day	8th day	10th day
P. vivax				
Expt. 1	A	97		63
	B	93		60
	C	93		47
	D	27		18
Expt. 2	A	93	83	—
	B	100	79	—
	C	100	79	—
	D	72	38	—
Expt. 3	A	100	83	—
	D	81	32	—
Expt. 4	A	100	87	—
	D	76	58	—
Expt. 5	A	100	70	—
	B	100	46	—
(treated at bedside)	D	52	14	—
P. falciparum				
Expt. 2	A	98	—	94
	C	100	—	85
	D	40	—	31
Expt. 4	A	99	90	—
	D	49	30	—

Note: vivax Expt. 2 received 0.0524 Mcgm D.D.T. All others 0.0638 Mcgm. Group A. Control, no treatment. Group B. CO_2 only. Group C. CO_2 plus acetone. Group D. Treated topically with DDT in acetone.

experiments as well as on two of the experiments using *P. falciparum*. The first census was taken on the first day following treatment. The second, to obtain a record of later survival, was taken on the day of dissections. This occurred on either the eighth or tenth day after the blood-meal. Later counts would have been valueless due to specimens removed for dissection. Table 1 presents the data taken on these counts.

Survival records were made primarily on the treated groups, D, and as a control on the untreated groups, A. In order to test the effect of the pre-treatment techniques, a few censuses were taken on the groups, B and C. During the first 24 hours mortality did not seem to be greatly affected by the treatment of these two groups, i.e., CO_2 and CO_2 plus acetone. However, some reduction in survival may be noticed at the second census where there seems to be a definitely deleterious effect.

The effect of the DDT is quite pronounced and, although somewhat masked by the effect of the CO_2 and acetone, is easily perceived. For instance, on the first day after treatment the three untreated groups, A, B, and C, ranged from 93 per cent to 100 per cent survival while group D given DDT had survival

range of from 27 per cent to 81 per cent. On the eighth and tenth days similar variations existed with the controls ranging widely between 46 per cent and 94 per cent. In all cases, survival where DDT was applied was considerably less than in the controls. This demonstrated that those mosquitoes that did survive had overcome a dose of insecticide that was fatal to many others in the group.

Effect of the insecticide on the Plasmodium. The effects of the topical treatments upon the ability of the *Plasmodium* to survive and develop in the mosquito

TABLE 2

Effect of topical application of DDT on the development of Plasmodium vivax *in* Anopheles quadrimaculatus

Experiment	Group	No. Dis.	No. Pos.	Per cent Pos.	Gut index	Gland index	Infective index
1	A	23	21	91	2.1	3.5	1,920
	B	18	18	100	1.9	2.8	1,380
	C	12	11	92	2.3	1.0	2,309
	D	6	6	100	2.2	—*	1,870
2	A	20	2	10	1.0	0.0†	50
	B	12	5	42	1.0	0.0	67
	C	12	4	33	1.0	0.0	33
	D	14	3	21	1.0	0.0	21
3	A	20	17	85	3.3	3.6	8,432
	D	10	10	100	3.4	0.0	11,610
4	A	20	19	95	3.4	3.8	18,810
	D	15	14	93	3.4	3.4	33,266
5	A	15	13	87	3.5	3.2	6,378
	B	11	11	100	3.0	—	6,170
	D	3	2	67	2.5	—	1,373

* No survivals for gland dissections.

† Glands negative.

Note: Experiment 2 received 0.0524 Mcgm. DDT. All others 0.0638 Mcgm. For group designations see Table 1.

TABLE 3

Effect of topical applications of DDT on the development of Plasmodium falciparum *in* Anopheles quadrimaculatus

Experiment	Group	No. Dis.	No. Pos.	Per cent Pos.	Gut index	Gland index	Infective index
1	A	26	21	81	2.2	2.2	2,033
	C	30	29	97	2.0	2.2	4,113
	D	24	23	96	2.7	1.3	4,618
2	A	10	8	76	2.2	1.8	1,687
	D	9	7	78	2.8	1.5	4,384
3	A	15	13	87	2.7	1.5	2,149
	D	7	7	100	2.0	1.3	1,760
	E	5	5	100	2.3	1.0	2,680
4	A	20	1	5	1.0	—	5
	D	18	5	28	1.0	—	28

Experiment 4 received 0.0524 Mcgm. DDT. All others received 0.0638 Mcgm. Group E treated four hours before blood meal. For group designations see Table 1.

were demonstrated by the data from the gut and gland dissections. Tables 2 and 3 present the results of dissections of the mosquitoes which had fed on malarious patients and had received topical applications of the insecticide. These tables also contain the records of dissection of the control group A as well as some from groups B and C which were run in order to determine the plasmodicidal effect of the pre-treatment techniques.

Both species of *Plasmodium* were able to reach the sporozoite stage in mosquitoes that had received a sublethal dose of DDT. Exposure of mosquitoes to this insecticide immediately or a few hours after ingestion of an infective blood meal appears not to affect the development of the parasite. In both Table 2 and Table 3 the treated group, D, often has a higher index of infection than the other groups.

In Table 3, experiment 3, group E, the insecticide was applied about four hours before the blood meal. Although only 21 per cent of the mosquitoes were able to feed, the parasite counts of the survivors compared favorably with those of the other treated groups. The survival of those that did feed was relatively high.

Although the treatment did not inhibit the development of the parasite, the surviving mosquitoes were left in such a clumsy, often partly legless, condition that they would probably have been destroyed before or during oviposition and therefore unable to take another blood meal. Since mosquitoes in control groups were not thus affected, it appears that a sub-lethal exposure to the drug is actually effective in interfering with transmission of malaria.

SUMMARY

Anopheles quadrimaculatus mosquitoes which had been exposed to infection with *Plasmodium vivax* or *P. falciparum* were topically treated with a sub-lethal dose of DDT in acetone. No lethal effect due to the insecticide was evident on the developing parasites. However, surviving mosquitoes were so adversely affected by the DDT that there seemed little chance they would successfully transmit the disease.

ACKNOWLEDGEMENTS

Acknowledgement is sincerely given to Dr. Richard W. Fay and Mr. Donald A. Lindquist of the Technical Development Laboratories, Technology Branch, Communicable Disease Center, Public Health Service, Savannah, Georgia, for determination of sub-lethal doses of insecticides and calibration of loops used in application as well as instructions in certain techniques.

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INFECTIVITY OF *PLASMODIUM BERGHEI* FOR *ANOPHELES QUADRIMACULATUS* AND OTHER MOSQUITOES[1]

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Since the discovery of *Plasmodium berghei* by Vincke and Lips in 1948, a considerable body of knowledge has been accumulated concerning the biology of this parasite, especially in regard to its development in vertebrate hosts. Less is known concerning the development of *P. berghei* in mosquitoes, which is the particular interest of the present investigation.

Although *Anopheles dureni* has been proven to be the natural vector of *P. berghei* (Vincke and Lips, 1950; Vincke and Peeters, 1953), experimental infection of other species of mosquitoes has been attempted by several workers in various laboratories. Raffaele and Baldi (1950) fed *Aedes aegypti* and *Culex pipiens*, two culicine species, on infected mice and rats but subsequent dissection failed to reveal oocysts or sporozoites of *P. berghei*. Jaswant Singh *et al.* (1951) did not obtain oocysts or sporozoites in 1,832 *Anopheles annularis* fed on mice, rats, and garden squirrels infected with *P. berghei*. Ramakrishman *et al.* (1953) failed to obtain infection with *Anopheles annularis*, *A. subpictus*, *A. culicifacies*, *A. splendidus*, *A. hyrcanus*, *A. jamesi*, *A. fluviatilis*, *A. pulcherrimus*, *Aedes aegypti*, *Culex bitaeniorhynchus*, and *Anopheles stephensi* fed on infected albino mice and rats, with the exception of one *A. stephensi* which was found to have a few sporozoites in its salivary glands. In 1951, Rodhain and Vincke were unsuccessful in attempts to infect *Anopheles maculipennis atroparvus* fed on splenectomized cotton rats, although a few immature oocysts were found in 2 of 65 mosquitoes fed on *Thamnomys surdaster* infected with the SP 28S Keilberg strain of *P. berghei*. Yoeli and Wall (1951) obtained a few viable sporozoites in the salivary glands of *Anopheles stephensi*, *A.m. atroparvus*, and *Anopheles quadrimaculatus* using the same strain of *P. berghei* in the golden hamster, and succeeded in infecting a second hamster with sporozoites from ruptured oocysts on the stomach wall of *A. stephensi*. The following year, these same investigators obtained a 50 per cent infection rate with *A. stephensi* and *A. quadrimaculatus* fed on a hamster infected with the Kasapa strain of *P. berghei*, and subsequently obtained a sporozoite-induced infection in a young rat inoculated with salivary glands of the above mosquitoes. However, in their transmission studies with the above species of anophelines, Yoeli and Wall (1951) mentioned that few sporozoites appeared able to reach the salivary glands. These authors noted Ross's black spores in the glands and gut from the tenth day after infection. Perez-Reyes (1953) fed *Anopheles aztecus*, *A. quadrimaculatus*, and *Anopheles albimanus* simultaneously on hamsters infected with the Mukata strain of *P. berghei*. Twenty-eight per cent of the *A. aztecus* became

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positive for both oocysts and sporozoites, while 3.3 per cent of the *A. quadrimaculatus* showed oocysts. One hundred fourteen *A. albimanus* were refractory to infection. Box *et al.* (1953) obtained numerous mature oocysts in *A. quadrimaculatus* fed on white mice and *Microtus p. pennsylvanicus* infected with the Kasapa strain of *P. berghei*. Although sporozoites developed abundantly within the oocysts, sporozoite-migration to the salivary glands failed to occur. The studies reported herein are a continuation of the work of Box *et al.*

MATERIALS AND METHODS

The Q_1 strain of *Anopheles quadrimaculatus* used in these studies was originally obtained from Dr. Richard W. Fay at Savannah, Georgia, and has been maintained in our insectary for several years. *Anopheles freeborni* eggs (F-1 strain originally from Marysville, California) were obtained through the courtesy of Dr. Robert W. Burgess at Columbia, South Carolina. *Anopheles bradleyi* adults were wild-caught in High Island, Texas, about 30 miles from Galveston, and transported to the laboratory. The *Aedes aegypti* were originally obtained from Dr. Richard W. Fay while the *Culex quinquefasciatus* were colonized by Dr. Don Micks from egg rafts collected in Galveston. Both species have been maintained at the Medical Branch for several years by Dr. Micks. All mosquitoes were reared in the usual manner, the larvae subsisting on powdered laboratory chow and Brewer's yeast, while the adults were maintained on raisins or four per cent sucrose solution. Three cages of *A. quadrimaculatus* were maintained, one for the stock mosquito colony and two to house the experimental mosquitoes to be infected.

The strain of *Plasmodium berghei* used throughout these studies was the Kasapa strain which was obtained through the courtesy of Dr. G. Robert Coatney at the National Institutes of Health. The strain was maintained by intravenous blood passage in female albino mice (Carworth Farms CF_1 strain) approximately every six days since the Kasapa strain usually kills mice in from six to ten days. White rats were obtained from the stock at the Medical Branch and weighed less than 100 grams when inoculated. Young hamsters, also weighing less than 100 grams, were from Joseph Stocker, Ramsey, New Jersey.

The mice to be used for experiments were inoculated intravenously with from 0.03 to 0.1 cc. of citrated heart blood from an infected donor mouse. Hamsters and rats were inoculated intraperitoneally with from 0.1 to 0.4 cc. of citrated heart blood, depending on the size of the animal. Blood samples of the infected animals were taken immediately before each experiment by snipping the animal's tail, fixing the smear in methyl alcohol and staining with two per cent Giemsa. The number of parasites per thousand erythrocytes was counted as well as the number of gametocytes per thousand red blood cells as an index of the animal's infection. Exflagellation preparations were made on tail blood before the experiments although there seemed to be little relation between exflagellation of the male gametocytes (tail blood) and the subsequent infectivity rates of the mosquitoes. The animal which was to serve as the infective host was anesthetized with sodium amytal and placed inside the cage of mos-

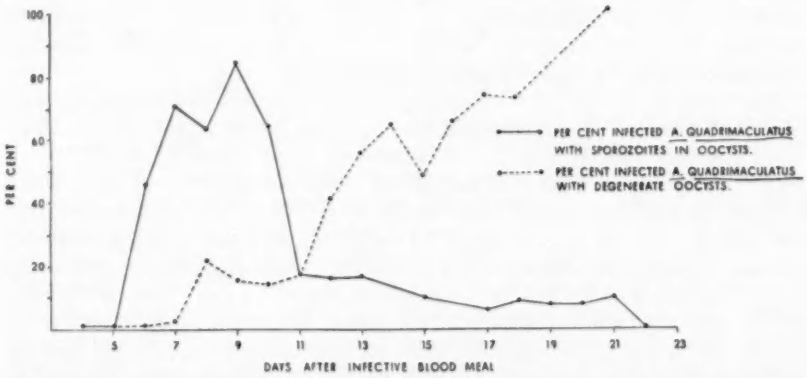
quitoes. After feeding, the engorged females were pipetted into lantern jars fitted to petri plates lined with moistened cellucotton, placed in a constant temperature chamber at 26°C. where they remained until dissection.

Unless they were to be used for animal inoculation, mosquito stomachs were dissected in 2.0 per cent mercurochrome as recommended by Eyles (1950). With this method, the oocysts are stained a deep red while the surrounding stomach tissue is stained pink, thereby facilitating oocyst detection. In those instances in which infected mosquito stomachs were to be inoculated into clean rodents in an attempt to infect them with sporozoites from oocysts, stomachs were dissected in physiological saline as were the salivary glands routinely. Using a microscope equipped with a 10× ocular and standard objectives (16 mm. and 4 mm.), the oocysts on each mosquito stomach were counted, measured with a micrometer and examined for the presence of sporozoites. Salivary gland dissections were done from the sixth day on after the infective blood meal.

RESULTS

In the hope of eventually obtaining sporozoite-induced infections of *Plasmodium berghei*, laboratory-reared *Anopheles quadrimaculatus* were fed on various rodent species with blood-induced infections of the Kasapa strain of *P. berghei*. Although mosquito infections with numerous mature oocysts on the stomach wall were obtained, sporozoites were never found in the salivary glands.

Development of P. berghei *oocysts in A. quadrimaculatus.* Development of oocysts in *A. quadrimaculatus* maintained at 26°C. was followed by dissection at intervals ranging from 4 to 21 days after a blood meal on infected white mice. Although a representative number of oocysts on each infected stomach was measured, no definite pattern of growth in terms of microns was apparent due to the tremendous variation in oocyst size, not only within a group of mosquitoes comprising an experiment but also within individual mosquitoes on various days



GRAPH 1. Loss of sporozoites from oocysts and degeneration of oocysts on stomachs of *A. quadrimaculatus* dissected at intervals after a blood meal on mice infected with *P. berghei*.

after the infective meal. In general, however, oocysts measured from 30 to 35 microns in diameter when mature, although a maximum size of 57.6 microns was attained by several oocysts. As shown in Graph 1, sporozoite development within oocysts becomes evident on the 6th day and increases until the 9th day following the infective blood meal. After this time the percentage of sporozoite-bearing oocysts falls with a concurrent rise in the percentage of degenerate oocysts. The degenerative changes include shrinkage, distortion or collapse, loss of sporozoites, and a granular appearance. Black spores were found within oocysts as well as scattered over the mosquito stomach in a number of instances eleven or more days after the initial infection.

In general, the percentage and intensity of the mosquito infections depended to a great extent on the rodent species utilized for the infective blood meal as well as the time the mosquitoes were fed with respect to the course of the parasitemia in the rodent host. During the first few days of the parasitemia, it was found that *A. quadrimaculatus* could be infected with *P. berghei* whether white mice, white rats, or hamsters were utilized as the infective host for mosquito feedings; however, the species of rodent did appear to exert a quantitative effect on the percentage and intensity of the infection in mosquitoes.

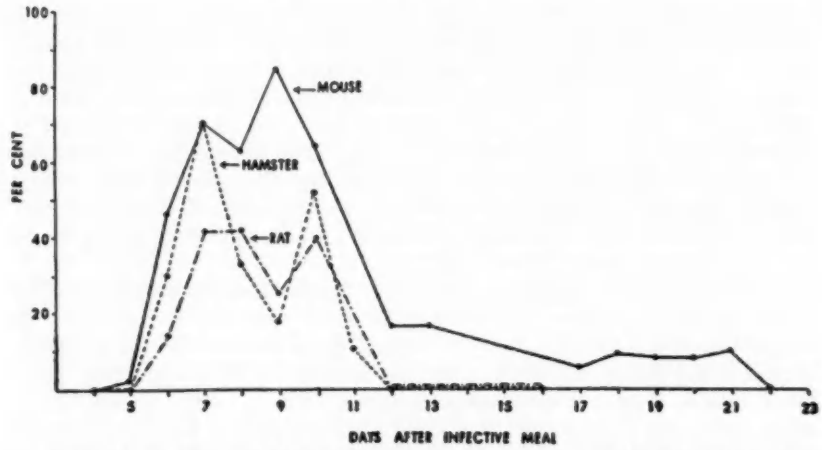
Effect of rodent host on percentages of mosquitoes infected. As shown in Table 1, of a total of 708 *A. quadrimaculatus* fed on various days of the malaria infection in white mice, 536 mosquitoes or 75.7 per cent were subsequently positive for oocysts. When white rats and hamsters were utilized as the infective hosts for mosquito feedings, lower infectivity rates were obtained. Of a total of 436 mosquitoes fed on white rats, 34.8 per cent became infected, while 33.1 per cent of 332 mosquitoes fed on hamsters were positive for oocysts (Table 1).

Effect of rodent host on numbers of oocysts. In addition to lower infectivity rates resulting when rodent species other than mice are used for the infective hosts, numbers and development of oocysts are apparently influenced also. Oocysts numbering in the hundreds and measuring up to 44.8 microns in diameter were not uncommon in mosquitoes fed on mice; while in those mosquitoes fed on rats and hamsters, oocysts were few in number and usually appeared somewhat shrunken and distorted. As shown in Table 1, 39.4 oocysts per stomach was the average number for mosquitoes fed on mice, a 5.9 oocyst average was found for those mosquitoes fed on rats, while 5.3 oocysts was the average for

TABLE 1

Comparison of Anopheles quadrimaculatus *fed on white mice, white rats, and hamsters with Kasapa strain of* Plasmodium berghei

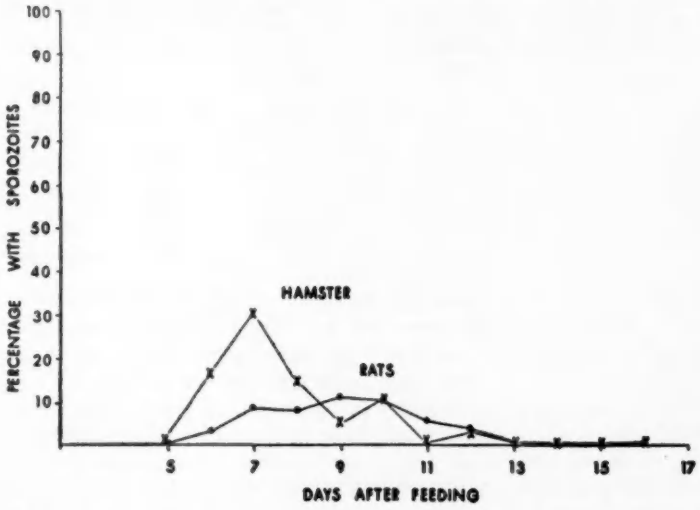
	Mice	Rats	Hamsters
Average no. gametocytes per 1000 rbc	8.0	8.8	5.0
Total no. of mosquitoes fed	708	436	332
Per cent of mosquitoes infected	75.7	34.8	33.1
Average no. oocysts per stomach of infected mosquito	39.4	5.9	5.3



GRAPH 2. Percentage of infected *A. quadrimaculatus* with sporozoites in oocysts after feedings on mice, rats, and hamsters infected with *P. berghei*.

mosquitoes fed on infected hamsters. Gametocytes were plentiful in all rodent species at the time of mosquito feedings, averaging 8.0 gametocytes per thousand erythrocytes in the mice, 8.8 per thousand erythrocytes in rats, and a 5.0 average per thousand erythrocytes in the hamsters at the time of mosquito feedings. It appears, therefore, that the gametocyte abundance factor was controlled sufficiently so that the difference in numbers of oocysts represents a definite qualitative difference of infective blood from the various species of rodent with the same species and strain of mosquito.

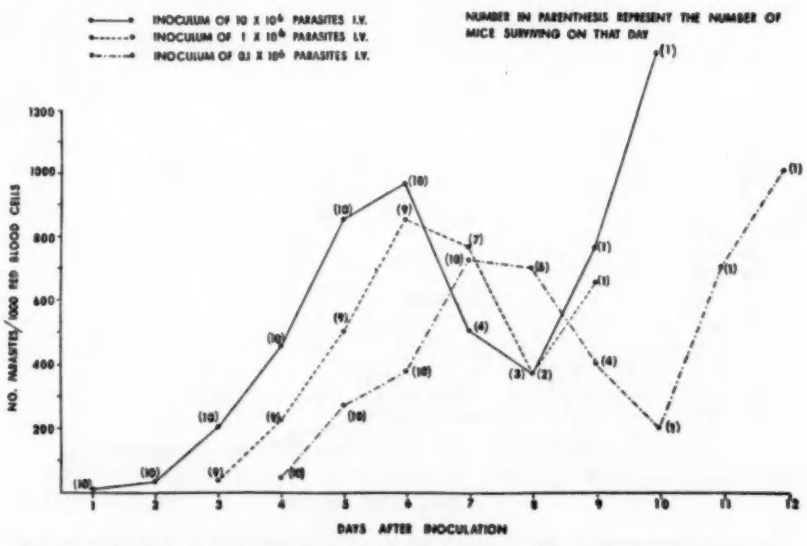
Effect of rodent host on development of oocysts. Development of oocysts within the infected mosquitoes also appears to be influenced by the source of the infective meal. Graph 2 shows that in mosquitoes fed on mice, development of oocysts to maturity, as evidenced by the presence of large numbers of sporozoites within the oocysts, is attained by the seventh to the ninth day. In dissections performed on the seventh day after the infective meal, 70.2 per cent of the mosquitoes had sporozoites within the oocysts, while the percentage fell to 63.4 per cent on the eighth day and rose to a peak of 84.2 per cent on the ninth day after which the numbers of mosquitoes with sporozoites in the oocysts steadily decreased on successive days. However, in mosquitoes fed on rats and hamsters and maintained at the same constant temperature of 26°C., oocyst development is frequently halted short of maturity since the percentage of infected mosquitoes whose oocysts contained sporozoites is consistently lower. Graph 2 shows that although the highest percentage of mosquitoes with sporozoites in oocysts was reached earlier in mosquitoes fed on rats and hamsters than when fed on mice, the percentage was consistently lower in the first two on all dissection days. There was also a noticeable difference in the numbers of oocysts containing sporozoites in individual mosquitoes when fed on mice as compared with those



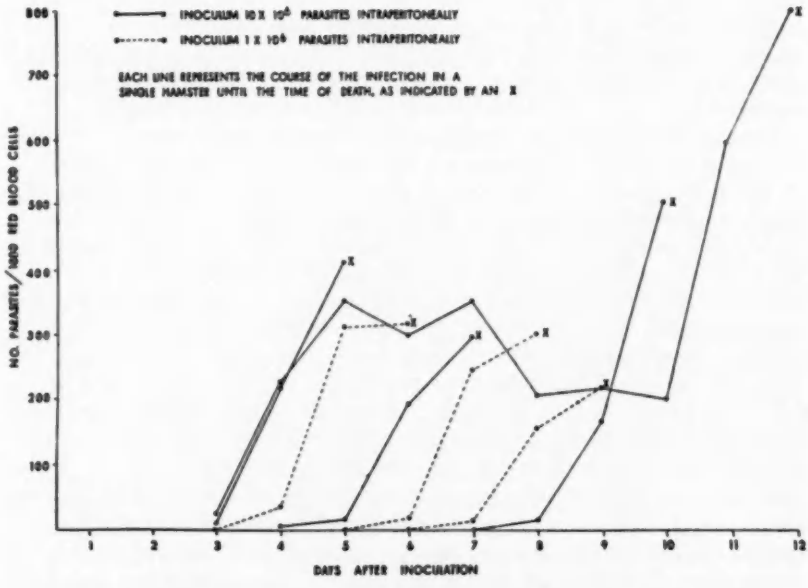
GRAPH 3. Percentage of oocysts in *A. quadrimaculatus* containing sporozoites on various days after feeding on infected rats and hamsters.

fed on the rat or hamster. Stomachs of those mosquitoes fed on mice often had sporozoites in 90 to 100 per cent of the oocysts when mature (and with oocyst counts numbering in the hundreds, were difficult to count). Conversely, Graph 3 shows that relatively few oocysts obtained from rat and hamster feedings ever contained sporozoites, the peaks being 11 and 30 per cent, respectively.

Course of blood-induced P. berghei *infection in mice and hamsters.* In order to determine the course of a blood-induced infection with the Kasapa strain of *P. berghei* in rodents, a small series of mice and hamsters was inoculated with known numbers of parasites after which the parasitemia was followed by daily blood smears until death of the animal occurred. As seen in Graph 4, although the group of mice receiving the smallest number of parasites, 0.1×10^6, did not have a demonstrable parasitemia until from one to three days later than the mice inoculated with 1×10^6 and 10×10^6 parasites, the average day of death was approximately the same for all three groups (7.3 days for the group receiving 10×10^6 parasites, 7.1 days for the 1×10^6 group of mice, and 8.6 days for the 0.1×10^6 group). In most instances, the mice died at the height of the infection with from 500 to 900 parasites per 1,000 erythrocytes, although a few mice survived the initial parasitemia peak and died at a second elevation. A tenfold difference in inoculum seemed to exert little influence on the duration and course of the parasitemia in hamsters inoculated intraperitoneally with ten million and one million parasites. As seen in Graph 5, all but one animal died within three days after the initial appearance of parasites in the peripheral circulation. In general, during the peak at which time death occurred, parasites


INOCULUM OF 10 X 10⁶ PARASITES I.V.
INOCULUM OF 1 X 10⁶ PARASITES I.V.
INOCULUM OF 0.1 X 10⁶ PARASITES I.V.
NUMBER IN PARENTHESIS REPRESENT THE NUMBER OF MICE SURVIVING ON THAT DAY
NO. PARASITES/1000 RED BLOOD CELLS
DAYS AFTER INOCULATION

GRAPH 4. Course of blood-induced *P. berghei* infection (Kasapa strain) in white mice.


INOCULUM 10 X 10⁶ PARASITES INTRAPERITONEALLY
INOCULUM 1 X 10⁶ PARASITES INTRAPERITONEALLY
EACH LINE REPRESENTS THE COURSE OF THE INFECTION IN A SINGLE HAMSTER UNTIL THE TIME OF DEATH, AS INDICATED BY AN X
NO. PARASITES/1000 RED BLOOD CELLS
DAYS AFTER INOCULATION

GRAPH 5. Course of blood-induced *P. berghei* infection (Kasapa strain) in hamsters.

TABLE 2

Influence of the age of malaria infection in mice on the degree of infectivity for Anopheles quadrimaculatus

Day of infection in mouse	Av. no. parasites/1000 r.b.c.	Av. no. gametocytes/1000 r.b.c.	No. mosq. fed	Per cent infected	Av. no. oocysts per stomach
2nd	109	6	243	95.8	57.2
3rd	253	8	293	87.4	63.3
4th	347	12	95	41.0	35.2
5th	561	7	77	10.4	3.0

numbered from 300 to 500 per thousand red blood cells, being considerably lower than the average parasitemia in the mice at the time of death.

Effect of age of malaria infection in rodents on the percentage of mosquitoes infected. The time of the infective blood meal for mosquitoes with respect to the peak of the parasitemia in the rodent host had a most pronounced effect on the percentage of mosquitoes which were subsequently infected. Considering the results of all experiments utilizing white mice as the infective host, as summarized in Table 2, 95.8 per cent of the *A. quadrimaculatus* which were fed on mice two days after inoculation with *P. berghei* became infected, and 87.4 per cent of those fed on the third day after the mice were inoculated were positive for oocysts. Thus, considering the infectivity percentages together obtained from all feedings on the second and third day after the mice were inoculated, 91.2 per cent of 536 *A. quadrimaculatus* fed during the early stages of the parasitemia were positive for oocysts of *P. berghei*. However, as shown in Table 2, when mosquito feedings were delayed until the fourth and fifth days after mouse inoculation, progressively fewer mosquitoes became infected, the percentages dropping to 41.0 and 10.4 per cent on the fourth and fifth days, respectively. A concomitant decline in average number of oocysts per infected stomach is seen on these two days as well. Feedings beyond the fifth day were quite difficult since the infected mice become moribund and die on the sixth or seventh day after inoculation.

In order to obtain a more accurate picture of the effect the age of the parasitemia exerts on the percentages of mosquitoes infected, several experiments were conducted on individual mice, rats, and hamsters by feeding the mosquitoes on a single animal on successive days of the infection. The infected animals were offered to the mosquitoes each day until the death of the animal, although mosquitoes were reluctant to feed on moribund, anemic animals in the terminal stages of the infection. As shown in Table 3, as the parasitemia progressively increased in all three rodent species, the number of mosquitoes positive for oocysts decreased although gametocytes were present in the peripheral circulation of all animals at the time of feeding experiments.

Attempts to obtain sporozoite-induced infections. Although oocysts of *P. berghei* were obtained with relative ease in *A. quadrimaculatus*, dissections of several hundred salivary glands of this species failed to reveal sporozoites. Therefore, several attempts were made to obtain sporozoite-induced *P. berghei* infections

TABLE 3

Effect of the age of the P. berghei *infection in various rodents on the infectivity rates of* A. quadrimaculatus

Host	Day	Parasites/1000 rbc	Gametocytes/1000 rbc	No. mosq. fed	No. mosq. infected	Per cent infected
Mouse #52	2nd	82/1000	9/1000	11	11	100.0
#52	4th	405/1000	16/1000	10	6	60.0
Mouse #59	3rd	276/1000	7/1000	12	12	100.0
#59	5th	598/1000	6/1000	5	0	0.0
Mouse #252	2nd	104/1000	4/1000	20	20	100.0
#252	3rd	216/1000	10/1000	23	23	100.0
#252	4th	416/1000	15/1000	23	11	47.8
#252	5th	573/1000	7/1000	21	1	4.7
Hamster #2	8th	53/1000	6/1000	17	14	82.3
#2	9th	115/1000	9/1000	17	10	58.8
#2	10th	264/1000	1/1000	5	3	60.0
Hamster #4	5th	26/1000	3/1000	51	26	51.0
#4	6th	132/1000	4/1000	30	9	30.0
#4	7th	166/1000	1/1000	25	5	20.0
Hamster #5	2nd	58/1000	4/1000	20	10	50.0
#5	3rd	257/1000	9/1000	23	2	8.7
#5	4th	413/1000	10/1000	22	1	4.5
#5	5th	268/1000	4/1000	20	0	0.0
Rat #4	4th	184/1000	9/1000	45	28	62.2
#4	5th	465/1000	13/1000	67	3	4.4
Rat #5	4th	67/1000	5/1000	33	22	66.6
#5	5th	154/1000	7/1000	37	29	78.3
#5	6th	287/1000	9/1000	33	6	18.1
#5	7th	402/1000	10/1000	38	2	5.2
#5	8th	469/1000	11/1000	41	0	0.0

in mice (splenectomized and non-splenectomized), rats, and hamsters by intravenous and intraperitoneal inoculation of *A. quadrimaculatus* stomachs, containing large numbers of mature oocysts, salivary glands, and finally entire mosquitoes. However, blood smears examined periodically for several weeks failed to reveal malaria parasites in the recipient animals. (It is of considerable interest that Yoeli and Wall (1951) did succeed in infecting a hamster with sporozoites from ruptured oocysts in *Anopheles stephensi* of the SP 28S Keiberg strain of *Plasmodium berghei*).

Susceptibility of other mosquito species to P. berghei. Since sporozoites of *P. berghei* failed to migrate to the salivary glands of *A. quadrimaculatus*, it was desirable to compare the susceptibility of various other species of anophelines to *P. berghei* to see if, in a suitable vector, sporozoites would migrate to the salivary glands. A few individuals of laboratory-reared *Anopheles freeborni* as well as several wild-caught *Anopheles bradleyi* were fed on rodents infected with *P. berghei* with the results shown in Table 4. With the exception of the first *A. bradleyi* experiment, *A. quadrimatulatus* mosquitoes were fed on the same animals as controls immediately after the *A. freeborni* and *A. bradleyi*. Unfortu-

TABLE 4

Comparison of the susceptibility of various mosquito species to the Kasapa strain of Plasmodium berghei

Mosquito Species	Host	Parasites/ 1000 rbc	Gametocytes/ 1000 rbc	Day	No. mosq. fed	No. mosq. infected	Per cent infected
A. bradleyi	Mouse #21	166/1000	3/1000	3rd	3	3	100.0
A. bradleyi	Mouse #36	116/1000	7/1000	2nd	6	5	83.3
A. quadrimaculatus	#36	116/1000	7/1000	2nd	20	20	100.0
A. freeborni	Mouse #243	167/1000	4/1000	2nd	7	6	85.7
A. quadrimaculatus	#243	167/1000	4/1000	2nd	12	2	16.6
A. freeborni	Mouse #243	386/1000	15/1000	3rd	8	3	37.5
A. quadrimaculatus	#243	386/1000	15/1000	3rd	8	0	0.0
A. freeborni	Hamster #5	58/1000	4/1000	2nd	16	2	12.5
A. quadrimaculatus	#5	58/1000	4/1000	2nd	20	10	50.0
C. quinquefasciatus	Mouse #32	116/1000	8/1000	3rd	42	0	0.0
A. quadrimaculatus	#32	116/1000	8/1000	3rd	17	17	100.0
Aedes aegypti	Mouse #44	117/1000	15/1000	3rd	32	0	0.0
A. quadrimaculatus	#44	117/1000	15/1000	3rd	9	9	100.0

nately, a valid comparison of susceptibility of the test mosquitoes with that of the control mosquitoes cannot be made due to the small numbers of test mosquitoes involved. However, it is evident that *P. berghei* will develop to the oocyst stage in both *A. freeborni* and *A. bradleyi* although gland infections were not obtained in either of the two species of mosquitoes.

Forty-two *Culex quinquefasciatus* and 32 *Aedes aegypti* were refractory to infection, while 100 per cent of the *A. quadrimaculatus* controls, fed immediately following the feeding of the culicines on mice, developed large numbers of oocysts.

DISCUSSION

The importance of the time of feeding with respect to the peak of the parasitemia in the rodent hosts has not been considered in all attempts to infect mosquitoes with *P. berghei* and may have a bearing on some reported negative results. However, the sharp decline in infectivity percentages of mosquitoes as the parasitemia in rodents progressed toward the peak demonstrated in our experiments was also noted by Perez-Reyes (1953) in his studies with *Anopheles aztecus* and *A. quadrimaculatus*. Furthermore, Perez-Reyes states in his article that his first attempts to obtain infections in anopheline mosquitoes were unsuccessful because feedings were made at and after the peak of the infection.

A number of workers experimenting with bird malaria have noted a lack of correlation between number of gametocytes circulating in the host and the subsequent percentage and intensity of mosquitoes infected (Lumsden and Bertram, 1940; Jeffery, 1944; Cantrell and Jordan, 1946; Micks, 1949; Eyles, 1952a; Huff and Marchbank, 1955). That the infectivity for mosquitoes does depend upon the gametocyte density—all other factors being equal—was well

demonstrated by Eyles (1951), using *in vitro* techniques. Eyles also found that "certain chickens were significantly more infective to mosquitoes than others, the difference being unrelated to gametocyte density". In our studies with *P. berghei*, the decline in mosquito infectivity percentages as the parasitemia progresses to a peak in the rodent hosts cannot be attributed to a drop in the relative gametocyte count, since the average number of gametocytes per thousand red blood cells was at a peak on the 4th day of patency in the mouse, whereas mosquito infectivity percentages were highest on the 2nd day. The estimates of gametocyte levels are, however, arbitrary, since the recorded number of parasites is in relation to the number of erythrocytes and not to the volume of blood. The severe parasitemia seen in mice inoculated with the Kasapa strain of *P. berghei* may, however, compare with severe *P. gallinaceum* infections in which Cantrell and Jordan (1946) and Eyles (1952a) found that as the infection reached a peak, the infectivity of the gametocytes for mosquitoes fell markedly.

Cantrell and Jordan (1946) demonstrated *in vivo* and Eyles (1952a) demonstrated *in vitro* that substitution of normal serum for serum from chickens severely parasitized with *P. gallinaceum* would significantly enhance the infectivity of the gametocytes for *Aedes aegypti*. In earlier studies, in which he worked with milder sporozoite-induced rather than blood-induced infection, Eyles (1951) did not encounter the tendency for gametocytes to become less infective for mosquitoes as the peak of the parasitemia was reached. Eyles remarked that if such a decrease in infectivity of gametocytes in severe blood-induced infections is attributed to the depletion of some essential factor in the blood, it is to be expected that the more benign sporozoite-induced infection with its fewer parasites, would not induce such a diminution and resultant decline in infectivity.

Few workers have studied the effect of the species of host on the infectivity for mosquitoes, even in avian malaria. In the studies of Hunninen *et al.* (1950) upon the susceptibility of various mosquitoes to *Plasmodium relictum*, it was found that they were more susceptible to the malaria infection in the sparrow than in the canary. Attempts to pass the infection from sparrow to canary by sporozoites failed until the infection had been established by blood-inoculation in the canary, after which it could be transmitted by mosquitoes to another canary. Redmond (1944) found that *P. relictum* of the pigeon could be established by mosquito transmission from canary to canary but not from canary to pigeon. Micks (1949) found that when *Culex pipiens* were fed on canaries infected with *P. elongatum*, sporogonous development proceeded only to the early oocyst stage; however, when this same species of mosquito received its blood meal from an infected duck, complete development of the plasmodium through the sporozoite stage occurred. In addition, higher infectivity rates as well as more intense oocyst infections resulted from feedings on the duck. Recently, Huff and Marchbank (1955) reported that infectivity of the gametocytes of *Plasmodium fallax* for *Aedes albopictus* was affected by the avian host as judged by the resulting number of oocysts. The guinea fowl, which is closely related to the host from which the parasite was originally isolated, proved to be a good

host in respect to the oocyst production as related to gametocyte numbers, while the chicken was a poor host.

In the three instances in which cyclical transmission of *P. berghei* has been achieved experimentally (Yoeli and Wall, 1951, 1952; Perez-Reyes, 1953), the anopheline mosquitoes with which sporozoite transmission occurred were all infected from hamsters. In their original experiment, Yoeli and Wall (1951) failed to find any evidence of infection in a large group of *Anopheles stephensi* fed on a white rat infected with *P. berghei*; however, when part of the surviving mosquitoes from this group were fed on an infected hamster, 73 per cent became positive. As no details concerning the stage of the parasitemia in the rodents are given, it is more likely that the negative results obtained from the rat feeding can be explained on the basis of time of feeding in relation to the parasitemia peak rather than an influence exerted by the species of host.

Our data, coupled with the observation of Yoeli and Wall, that few sporozoites appeared able to reach the salivary glands in their experiments, indicate that although development of *P. berghei* proceeds normally up to the oocyst stage in *A. quadrimaculatus*, the oocysts tend to degenerate about the time one would expect migration of the sporozoites to the salivary glands. This inability of *P. berghei* successfully to complete sporogonous development within *A. quadrimaculatus* and other species of anophelines, may parallel the partial development of certain species of avian malaria in anopheline mosquitoes. Although the natural hosts of avian malaria are culicine mosquitoes, several investigators have reported infections of anopheline mosquitoes with various species of bird malaria. The percentage of anophelines infected is low as compared with culicine mosquitoes, and sporozoites, with a few exceptions, are rarely found in the salivary glands. Eyles (1952b) obtained heavy stomach infections of *P. gallinaceum* in *A. quadrimaculatus* as well as a few gland infections, although the number of sporozoites which migrated to the salivary glands was not proportionate to the number of oocysts on the mosquito stomachs. The same investigator also obtained light stomach infections of *P. gallinaceum* in *Anopheles freeborni* but no gland infections. Using another species of avian malaria, *P. cathemerium* in the canary, Micks and McCollum (1952) obtained large numbers of oocysts but no gland infections in the Q1 strain of *A. quadrimaculatus*, the same strain and colony as was used in our *P. berghei* studies, although for several years this strain of mosquito was refractory to infection with the same strain of *P. cathemerium*.

The failure of sporozoites of the Kasapa strain of *P. berghei* to accomplish migration from oocysts to the salivary glands in the Q1 strain of *A. quadrimaculatus* as in the case of certain anophelines infected with avian malaria may be due to lack of an essential nutriment or to the presence of a deleterious substance which prevents migration of sporozoites in the hemolymph of the mosquito. Developmental failure apparently may occur at any point in the sporogonous cycle from exflagellation (Micks *et al.*, 1948) and penetration of the ookinete through the stomach wall (Huff, 1932) through migration of the sporozoites to the salivary glands. Terzian *et al.* (1949), in studies conducted on the effects

of various antimalarials on the sporogonous cycle of *P. gallinaceum*, was able to show that the metabolic requirements of the parasite apparently change as it proceeds from stage to stage in its developmental cycle, since compounds effective at one specific stage had no effect at another. In addition, Terzian *et al.* (1952, 1953) demonstrated that certain biochemical compounds including antibiotics, vitamins and hormones when administered in specific concentrations, may exert an influence on the innate immunity of various mosquito species to *P. gallinaceum*, some enhancing and others decreasing susceptibility. These investigators found that drug concentration in itself is an extremely critical factor in determining the effect exerted on the immunity of the mosquito host to the parasite. Furthermore, such influences are specific for a particular host-parasite relationship; *i.e.*, a compound which increases the susceptibility of *A. aegypti* to *P. gallinaceum* at one concentration may increase the resistance of *A. quadrimaculatus* to the same parasite when administered at the same concentration.

In view of the above findings on *P. gallinaceum*, it would be of interest to investigate the ability of various substances quantitatively to influence mosquito infectivity rates obtained from feedings on infected hamsters and rats in which relatively low percentages of infection with *P. berghei* are obtained.

At the present stage of investigation there is no readily cultivated mosquito with a sufficiently high degree of susceptibility of *P. berghei* to serve as a laboratory vector. *Anopheles dureni* has not been cultured in the laboratory, and none of the anophelines in which complete sporogonous development has been reported is suitable for consistent sporozoite transmission of the parasite. *Anopheles aztecus* adults do not readily survive for a period sufficient for sporozoite migration to occur, and gland infections obtained in *A. m. atroparvus* and *A. stephensi* are reported as being irregular and sparse. The development of *P. berghei* in *A. quadrimaculatus* presents an interesting problem because of a very high degree of susceptibility of the mosquito to oocyst development. One may suspect some factor (possibly in hemolymph) which is inhibitory to proper migration of the sporozoites to the salivary glands. However, the inability of macerated sporozoite-bearing oocysts to induce infections in rodents may indicate failure of complete development of the sporozoites within the oocysts. Although the literature as well as the experiments reported here include a number of details which influence development of *P. berghei* in the mosquito, none of them touches on the barrier to complete development and migration of sporozoites which are abundant in oocysts. The latter has a bearing on an understanding of the mechanism of susceptibility and non-susceptibility of mosquitoes to malarial parasites and suggests the possibility of further experimentation with sporozoite-induced infections in laboratory animals.

SUMMARY

At a temperature of 26°C. oocysts of *P. berghei* in *A. quadrimaculatus* fed on infected white mice contained sporozoites by the 6th day, with a peak number of oocysts containing sporozoites on the 9th day. The decrease in proportion

of sporozoite-bearing oocysts after this time indicated that migration of the sporozoites to the salivary glands would normally take place at this time. However, infected glands were never found. Development of *P. berghei* to the oocyst stage was also obtained in two additional species of *Anopheles* not previously reported. Two species of culicine mosquitoes showed no evidence of infection with *P. berghei*.

Attempts to obtain sporozoite-induced infections in mice, rats, and hamsters by intravenous and intraperitoneal inoculation of stomachs with large numbers of mature oocysts, salivary glands or entire mosquitoes were unsuccessful.

The rodent host used as a source of the infecting blood meal appeared to be an important factor in the percentage of mosquitoes infected and oocyst development. A higher percentage of *A. quadrimaculatus* became infected and the number of oocysts per infected mosquito stomach was greater when mice were used as the source of the infecting meal than was the case when the same species of mosquito was fed on rats or hamsters. In addition, sporozoites developed more readily in mosquitoes whose infective blood source was mice.

The time of feeding with respect to the parasitemia peak in the rodent host had a most pronounced effect on the percentage of mosquitoes infected. Rodents in the early stages of the disease were more infective for mosquitoes than were rodents at or past the parasitemia peak.

The course of the Kasapa strain of *P. berghei* was determined for a small series of mice inoculated intravenously and hamsters inoculated intraperitoneally with a counted number of parasites. With 3 different sizes of inoculum the patency of the infection in white mice was delayed with the smallest dose, although the average day of death was about the same for the three groups. A ten-fold difference in inoculum seemed to exert little influence on the duration and course of the parasitemia in intraperitoneally inoculated hamsters.

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THE LOSS AND REDEVELOPMENT OF INSECTICIDE RESISTANCE IN EGYPTIAN HOUSE FLIES[1]

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The Egyptian house fly, *Musca domestica vicina*, is capable of developing marked resistance to chlorinated hydrocarbon insecticides when these materials are used continuously (Gahan and Weir, 1950; Madwar and Zahar, 1951; Weir *et al.*, 1952). The resistance problem became so serious in 1952 that officials in the Egyptian Ministry of Health recommended that no chlorinated hydrocarbons be used for general house fly control.

STUDIES OF RESISTANCE

In 1953 a study was made to determine the resistance of *M. d. vicina* populations in areas from which information on previous insecticide usage was available and where little or no insecticide had been used for some time. It was considered that results of such a study would be helpful in determining which insecticides might be useful in an emergency. Flies from 11 localities in or near Cairo were tested for susceptibility to DDT, lindane, pyrethrins, and parathion by the topical-application method.

SOURCES OF FLIES

Flies were obtained from the Abbassia dump and Cairo slaughterhouse, both in Cairo, the Heliopolis slaughterhouse in a suburb, and eight rural localities between 8 and 20 miles from the center of the city.

The Ministry of Health and the International Health Division of the Rockefeller Foundation had made extensive insecticide studies in Aghour el Sughra, Konayesa, Quaranfil, Sindbis, and Tersa from 1948 to 1951. In these villages frequent applications of DDT, BHC, and chlordane were made to walls, floors, or fly-breeding places. The formulations used each year are given in Table 1.

A laboratory strain of flies established from field strains in 1947 and maintained since that time in the absence of insecticides at the U. S. Naval Medical Research Unit No. 3, Cairo, was used as a standard in all tests.

METHODS

The microsyringe used to apply solutions of insecticides to immobilized flies was similar to one in use at the Corvallis, Oregon laboratory of the Entomology Research Branch. It consisted of a ¼-ml. tuberculin syringe and part of a

[1] The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

[2] On assignment from the United States Department of Agriculture, Agricultural Research Service, Entomology Research Branch.

TABLE 1

Insecticides used in 11 localities in or near Cairo, 1948–1952

Locality and year	Formulation and number of applications
Abbassia dump 1947	DDT aerial spray
Aghour el Sughra	
1948	DDT Em 1
1949	DDT Em 3, BHC dust 1
1950	BHC dust 2, chlordane WP 3
1951	Chlordane Em 3, BHC WP 2
Ashtar and Barada	Check villages, no insecticide
Cairo slaughterhouse 1951	BHC soln. 9, pyrethrins soln. 3
Heliopolis slaughterhouse 1949	BHC WP 1
Konayesa	
1949	BHC soln. 12
1950	BHC soln. 13, BHC dust 10
1951	BHC WP 2, chlordane WP 13, pyrethrins soln. 2
1952	BHC WP 27
Moneeb	
1949	DDT WP 1
1950	DDT WP 2, chlordane Em 1
Quaranfil	
1948	BHC dust 2
1949	BHC dust 11
1950	Chlordane WP 4
1951	BHC WP 3, chlordane Em 3
Sindbis	
1948	DDT Em 1, BHC dust 2
1949	BHC dust 12
1950	Chlordane WP 4
1951	Chlordane Em 3, BHC WP 2
Tersa	
1949	DDT Em 1, BHC WP 1
1950	BHC WP 3
1951	BHC WP 4, pyrethrins soln. 1

micrometer caliper mounted on a block of wood. A brass ring 1 inch in diameter, having equidistant cross grooves, was fastened on the knurled movable part of the micrometer handle. The ring was in constant contact with a horizontal spring wire. As the handle was turned, and each time the ring moved from one groove to the next on the wire, the plunger of the syringe was advanced a constant distance. Uniformity of delivery was checked by weighing volumes of acetone delivered when the end of the plunger was at different places in the tube of the syringe. Weighings were made in small glass tubes having an opening at one end just large enough to permit the insertion of a 27-gage needle. Although delivery was uniform for a given syringe, it varied with different syringes. Adjustments for these differences were made by varying the concentration of insecticide in the test solutions.

Fly densities in nature were low when this study was begun; therefore, fly strains were colonized and the F_1 generations tested. Later, when flies were more

numerous, field-collected specimens were tested. Tests comparing field-collected flies and the F_1 generation of the same strain yielded almost identical LD-50's (dosage required to kill 50 per cent of the flies) with these four insecticides. Colonized strains were tested when 4 to 7 days old, and field-collected flies 1 to 5 days after collection.

Group of flies were immobilized with carbon dioxide and females sorted from males. Each female received a topical application on the mesothorax of an acetone solution of one of the following insecticides: Technical DDT, lindane, a 20-per cent pyrethrum concentrate, or 96 per cent of parathion. Tests were repeated two to four times, and 20 flies were used per test. Each lot of flies was placed in a 3- by 9-inch screen cage provided with a cotton pad soaked with 10 per cent honey solution. Mortality counts were made after 24 hours.

DISCUSSION OF RESULTS

The "LD-50's" of the flies from each locality, computed by the method of Litchfield and Wilcoxon (1949), are shown in Table 2. None of the field strains were seriously resistant to any of the four insecticides.

With DDT and parathion, all field strains had somewhat higher "LD-50's" than the laboratory strain, and strains from the two slaughterhouses and the village of Tersa were more resistant to DDT than any of the other strains. The differences with parathion were very small.

With lindane, only Sindbis flies showed marked resistance. The flies from Aghour el Sughra, where insecticide applications had been made, were even more susceptible than specimens from Ashtar and Barada, the check villages.

With pyrethrins, only three of the field strains had higher "LD-50's" than the laboratory strain, and these differences were only slight. Flies from Aghour el Sughra and Quaranfil were the most susceptible.

On the basis of the statement of Decker and Bruce (1952) that five- to ten-fold resistance is required for significance, there was no marked resistance to the

TABLE 2

LD-50's of several insecticides to flies from 11 localities near Cairo and of a laboratory colony.
(Micrograms per fly)

Locality	DDT	Lindane	Pyrethrins	Parathion
Abbassia dump	2.7	0.14	0.98	0.016
Aghour el Sughra	1.6	.06	.36	.011
Ashtar	2.7	.08	.68	.017
Barada	2.2	.09	.67	.010
Cairo slaughterhouse	4.5	.14	.7	.016
Heliopolis slaughterhouse	3.6	.2	1.5	.010
Konayesa	2.6	.19	.77	.016
Moneeb	2.7	.28	1.1	.017
Quaranfil	1.8	.12	.45	.012
Sindbis	2.2	.46	.72	.011
Tersa	3.3	.15	.88	.016
Laboratory	1.0	.19	.96	.009

TABLE 3

LD-50's of several insecticides to flies from various regions. (Micrograms per fly)

Locality	DDT	Lindane	Pyrethrins	Parathion	Source
California	0.033	0.010	1.0	0.015	March and Metcalf (1949)
Florida	.12	.015	0.48	—	King (1950)
Illinois	.33	.03	—	—	Decker and Bruce (1952)
Egypt	1.0	.19	.96	.009	

four insecticides among the strains of Egyptian flies tested. Comparative "LD-50's" from several sources in the United States, shown in Table 3, indicate that Egyptian house flies are more resistant to DDT than normal flies from Florida and California, more resistant to lindane than flies from these two areas and Illinois, and more resistant to pyrethrins than flies from Florida. However, differences in technique and laboratory conditions may be responsible for the minor differences shown.

EFFECTIVENESS OF DUSTING FLY-BREEDING AREAS

Although Gahan and Weir (1950), Madwar and Zahar (1951), and Weir *et al.* (1952) showed that serious resistance to BHC and chlordane existed from 1949 to 1951, it appears from this study that by 1953 resistance had been lost. Other investigators have reported that field strains of resistant flies reared in the laboratory for several generations in the absence of insecticides have shown a similar reversion to susceptibility (Harrison, 1950; Upholt, 1950; King, 1950; Pimentel *et al.*, 1950 and 1953; March and Metcalf, 1949). Therefore in the summer of 1953 experiments were undertaken to evaluate the effectiveness of insecticide treatments in Konayesa, where BHC and chlordane had been lavishly used from 1949 to 1952, but where no treatments had previously been made in 1953.

A BHC dust containing 1.3 per cent of the gamma isomer was applied with small hand dusters to all potential breeding areas. One application gave a deposit of 100 mg. of active ingredient per square meter. The village was dusted once a week for 6 weeks from the middle of June until the end of July. Pre- and post-treatment fly counts made with a Scudder grill in Konayesa and in an untreated check village, Talibia, are shown in Table 4.

In order to determine whether resistance to BHC was increased, topical applications of lindane in acetone were made to female flies collected in Konayesa before and at various periods after the first dusting. The mortalities after 24 hours are shown in Table 5.

The fly population in Konayesa was reduced during the first 2 weeks after treatment, but satisfactory control was not achieved. By the third week the fly population was increasing in this village while in the untreated village it was decreasing. A downward trend of the Egyptian fly population is normal at this time of the year. The fly density in Konayesa exceeded that in Talibia until the middle of October, when it became greater in Talibia.

The dosage of lindane required to give 50-per cent mortality of Konayesa flies before treatment with BHC was almost identical with that for the laboratory

TABLE 4

Weekly fly densities in Konayesa (treated) and Talibia (untreated), as determined by average counts on a Scudder grill, 1953. In Konayesa BHC dust applied weekly from June 21 to July 30

Date		Konayesa	Talibia
June 1–4		356	317
June 6–10		282	252
June 15–18		223	177
June 21–25	BHC dust applied	210	240
June 26–July 1		177	226
July 4–9		192	195
July 11–16		250	181
July 18–23		267	124
July 25–30		263	123
August 1–6		130	91
August 8–12		156	83
August 15–18		162	75
August 24–27		166	61
August 29–September 3		192	49
September 5–9		167	157
September 12–17		162	105
September 19–24		157	139
September 26–October 1		152	120
October 3–8		159	151
October 10–15		182	166
October 17–22		161	236
October 24–29		154	158
October 31–November 5		172	231

TABLE 5

Per cent mortality from topical applications of lindane in acetone to house flies collected in Konayesa before and after treatment with BHC

Lindane (micrograms per fly)	Pre-treatment	Post-treatment		
		2–4 weeks	7 weeks	21 weeks
2.25	—	90	90	95
1.125	91	67	71	94
0.562	73	49	44	85
.281	56	30	28	53
.140	40	13	3	35
.070	34	—	—	23
LD-50 (micrograms per fly)	0.19	0.55	0.66	0.2

strain, but during the first 7 weeks this dosage increased at least threefold. This increase does not appear to be very serious, but Table 4 shows that frequent light applications of BHC did not control the flies in this village, although the same treatment had been effective in 1949 and 1950.

Heavier applications of BHC or other chlorinated hydrocarbons having long residual action may effectively control flies in Konayesa. However, once re-

sistance to one of these materials develops, resistance to the others develops rapidly (Simmons, 1954). It may be advisable to utilize insecticides such as the organic phosphorus compounds, to which neither rapid increase nor a great degree of resistance has been shown to develop in strains of flies in the United States. Those having low mammalian toxicities, such as malathion or tetrapropyl dithiopyrophosphate (NPD) may be used as residual treatments or in baits. New techniques may be necessary to make safe the use of some of the more toxic phosphorus compounds, such as parathion and EPN, around human and animal habitations.

SUMMARY

In 1952 the Egyptian Ministry of Health regarded the Egyptian house fly, *Musca domestica vicina*, as developing such serious resistance to the chlorinated hydrocarbon insecticides that they recommended the discontinuance of such materials for general house fly control. A study was made to determine the resistance of house flies from localities where insecticides had been used for several years but where no treatment had been undertaken for at least one fly season. Flies from 11 localities in or near Cairo were tested with topical applications of acetone solutions of DDT, lindane, pyrethrins, and parathion. From the LD-50's computed from 24-hour mortalities, none of the strains tested showed a high degree of resistance.

Six weekly applications of BHC dust containing 1.3 per cent of gamma isomer to breeding places in one village where a similar treatment had previously been effective did not control the flies, and by the seventh week after the initial treatment resistance had increased at least threefold. These results indicate that with the cessation of treatments with chlorinated hydrocarbon insecticides Egyptian house flies lose resistance, but that resistance rapidly increases with reapplications of these materials. It is therefore recommended that other types of insecticide or techniques be employed to control this serious pest.

ACKNOWLEDGMENTS

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EFFICACY OF THE CLOTHING IMPREGNANTS M-2065 AND M-2066 AGAINST TERRESTRIAL LEECHES IN NORTH BORNEO

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In certain parts of the world terrestrial leeches are serious pests that tend to limit human activity and the economic development in extensive areas. It has been reported by Traub *et al.* (1952) and Audy and Harrison (1954) that clothing impregnated with U. S. Army M-1960 insect repellent gave good protection against these blood-sucking annelids. The present paper gives the results obtained by a joint U. S. Army-British Colonial Office Medical Research Unit team[1], operating in North Borneo during the summer of 1953. Tests were made on two new experimental repellents, M-2065 and M-2066, and their efficiency in repelling terrestrial leeches was compared with that of M-1960.

MATERIALS AND METHODS

The clothing impregnants used in these tests consisted of the compounds M-1960, M-2065 and M-2066. The first is a standard item of issue for the Army under the name "Repellent, insect, clothing-treatment, M-1960." It consists of:

N-butylacetanilide	30%
2-butyl-2-ethyl-1,3-propanediol	30%
Benzyl benzoate	30%
"Tween 80" (Emulsifier)	10%

M-2065 and M-2066 are experimental mixtures that were developed by the U. S. Department of Agriculture in an Army-sponsored project, and have been found effective against mosquitoes, mites, ticks and fleas. These compounds have nothing in common with M-1960 except the emulsifying agent. As shown below, they differ from one another only in the amount of lindane they contain.

	M-2065	M-2066
Undecylenic acid	29%	29.66%
N-propylacetanilide	29%	29.66%
N-butyl-4-cyclohexene-1,2-carboximide	29%	29.66%
Lindane	3%	1.00%
"Tween 80" (Emulsifier)	10%	10.00%

For the tests, 1 part of each of the compounds was diluted with 11 parts water, and the clothing was impregnated by hand at the rate of 1 gallon of the concentrate for 28 uniforms. In order to avoid possible toxic effects to the wearer, the treated uniforms, after being thoroughly dried, were washed once with soap

[1] Supported in part by funds from the Research and Development Division, Office of the Surgeon General, Department of the Army, Washington.

and water, then rinsed 3 times in warm water before they were issued to the volunteers. The untreated uniforms, worn by volunteers serving as controls, were similarly washed to eliminate the factor of the size in the new cloth. The repellency of all the uniforms was tested after this initial washing, and then again after additional washings with soap and tepid water.

The methods used in the experiments were similar to those of Audy and Harrison (1954). Twenty indigenous volunteers were employed for the tests. Five persons wore clothes impregnated with M-1960; five wore clothing impregnated with M-2065 or M-2066; five wore untreated clothes; and five were attired in native dress. The clothes in all but the last group were standard Army issue tropical uniforms consisting of lightweight jacket and trousers. All persons wearing the uniforms were also furnished with quarter-sleeve undershirts and cotton-rayon socks, and in the case of those wearing treated uniforms these garments were also impregnated with the corresponding compound. Trouser legs were worn tucked into the top of the socks and the footwear consisted of the openwork rubber sandals commonly worn in this area. The native dress consisted only of a light shirt and shorts, with the legs and feet bare.

Trials were also made to test the effectiveness of treated socks alone as a means of protection for the scantily clad indigenous people. In these experiments five persons were barefooted, five wore untreated socks, five had socks treated with M-1960, and five had socks treated with M-2066. All but the first group wore the openwork rubber sandals. The socks, which reached almost to the knees of the short-statured Dusuns, were held in place with improvised garters of rattan.

Because of the spotty distribution of the leeches, and to insure comparable exposure, the volunteers were separated into five teams of four persons each. Each of the four types of clothing were represented on the teams. During the 30-minute exposure periods the various teams were sometimes rather widely separated. The members of each team, however, kept close together as they moved slowly along trails or cut paths through the underbrush. The leeches on the clothing and bodies of the volunteers were counted at the conclusion of each exposure period of 30 minutes. The teams were exposed in similar terrain two or three times during the course of each experiment.

OBSERVATIONS ON THE HABITATS AND HABITS OF THE LEECHES

The site of the tests was at Paring, North Borneo, approximately eight miles northeast of Ranau at an elevation of 1,500 feet. In this area the primary rain forest had been partially cut over and was secondarily overgrown with a heavy underbrush. There was a dense layer of leaf litter underfoot. Two distinct species of leeches were abundant and present in approximately equal numbers. One was the bush-climbing *Haemadipsa picta* and the other was the ground-dwelling *H. zeylanica subagilis*. A small number of a third type, apparently a color variant of *H. zeylanica*, were also found in the area. As noted by Audy and Harrison (1954), *H. zeylanica* attacked from the ground and usually attached below the knee of the host. A favored spot seemed to be between the toes of the barefooted

Dusun aborigines. By squeezing only the anterior end of the body through the mesh of the cloth, this species had little difficulty in penetrating and feeding, in spite of the finely woven cotton-rayon socks that had been issued to the group. The bush-climbing *H. picta* was observed to ascend only during or immediately following a rainfall, when the shrubbery was wet and the relative humidity approached 100 per cent. These leeches usually made contact with the host from vegetation at knee to waist level. Occasionally they were found to attach to persons from leaves at head level. When human beings approached their vicinity these leeches assumed a "searching" attitude. The caudal sucker remained attached but the leech elongated to more than twice its normal length and the anterior end waved back and forth. At the slightest contact with the victim, the extended leech would release the posterior sucker, and immediately attach to the host. None were ever seen to drop on their prey from above, as has been reported by other writers. However, it is not difficult to understand how a victim might erroneously conclude that a leech had gauged its fall to land on him as he passed. This would be especially true where he had been walking rapidly and the leech had been attached to vegetation at head level.

TABLE 1

Numbers of leeches per volunteer wearing impregnated or untreated clothing

Method of testing				Results			
				Numbers of leeches			
New type of compound and clothing	Number of washings in soap and water	Number of replications		New compound	M-1960	Untreated uniforms	Native dress
I. Complete uniform impregnated with M-2065	A. One: followed by three rinses in water	3	Total.....	1	0	77	48
			Range.....	0–1	0	9–27	9–13
			Mean.....	—	0	15.4	9.4
	B. As in "A" but with four additional washings	2	Total.....	15	16	70	54
			Range.....	1–6	1–9	4–32	10–12
			Mean.....	3.0	3.2	14	10.8
	C. As in "A" but with six additional washings	2	Total.....	24	9	37	53
			Range.....	1–12	0–3	5–10	8–13
			Mean.....	4.8	1.8	7.4	10.6
II. Complete uniform impregnated with M-2066	D. One: followed by three rinses in water	2	Total.....	2	2	71	114
			Range.....	0–1	0–1	8–24	22–26
			Mean.....	0.4	0.4	14.2	22.8
	E. As in "D" but with six additional washings	2	Total.....	37	12	32	63
			Range	4–11	0–9	2–10	10–15
			Mean.....	7.4	2.4	6.4	12.6
III. Only cotton-rayon socks impregnated with M-2066	F. One: followed by three rinses in water	2	Total.....	1	1	36	71
			Range.....	0–1	0–1	4–10	12–16
			Mean.....	0.2	0.2	7.2	14.2

TABLE 2

Average number of leeches attaching in thirty minutes to volunteers wearing impregnated or untreated clothing

Method of testing			Results			
New type of compound and clothing	Number of washings in soap and water	Number of replications	Average number of leeches			
			New compound	M-1960	Untreated uniforms	Native dress
I. Complete uniform impregnated with M-2065	A. One: followed by three rinses in water	3	0.06	0	5.1	3.2
	B. As in "A" but with four additional washings	2	1.5	1.6	7.0	5.4
	C. As in "A" but with six additional washings	2	2.4	0.9	3.7	7.1
II. Complete uniform impregnated with M-2066	D. One: followed by three rinses in water	2	0.2	0.2	7.1	11.4
	E. As in "D" but with six additional washings	2	3.7	1.2	3.2	6.3
III. Only cotton-rayon socks impregnated with M-2066	F. One: followed by three rinses in water	2	0.1	0.1	3.6	7.1

It was also noted that the distribution of the leeches in an infested area was quite irregular. Small foci, several square meters in extent, had large numbers of leeches while the intervening territory had only sparse populations.

RESULTS

Excellent protection from leeches was afforded by all three repellents after the initial washing, as shown in Tables 1 and 2. A maximum of one leech was found on protected individuals. In contrast, those wearing untreated uniforms averaged more than 14 leeches each, while those in native dress averaged more than 9 in one series of experiments, and more than 22 in another.

After four additional washings with soap and tepid water, M-1960 and M-2065 retained some of their effectiveness. In this experiment the average number of leeches on the persons wearing the treated clothing was less than one-fourth of those observed in the control groups, but protection was no longer complete and individuals in both protected groups suffered leech bites. M-2066 was not tested in this series but after a total of six washings this compound was completely ineffective. The number of leeches present on the volunteers wearing uniforms treated with M-2065, and washed six times, was only slightly less than the number found on the controls. M-1960 appeared to be somewhat more effective, but the numbers observed indicated that after six washings this compound was of no practical value.

It was noted that many of the leeches found on the individuals in these experi-

TABLE 3

Total numbers of fed and unfed leeches per group of volunteers wearing impregnated or untreated clothing

Method of testing				Results			
				Numbers of leeches			
New type of compound and clothing	Number of washings in soap and water	Number of replications	Condition of leeches	New compound	M-1960	Untreated uniforms	Native dress
I. Complete uniform impregnated with M-2065	A. One: followed by three rinses in water	3	Unfed	1	0	43	1
			Fed	0	0	34	47
	B. As in "A" but with four additional washings	2	Unfed	11	11	50	0
			Fed	4	5	20	54
	C. As in "A" but with six additional washings	2	Unfed	18	7	20	2
			Fed	6	2	17	51
II. Complete uniform impregnated with M-2066	D. One: followed by three rinses in water	2	Unfed	2	2	32	8
			Fed	0	0	39	106
	E. As in "D" but with six additional washings	2	Unfed	25	5	19	5
			Fed	12	7	13	58
III. Only cotton-rayon socks impregnated with M-2066	F. One: followed by three rinses in water	2	Unfed	0	1	12	1
			Fed	1	0	24	70

ments were unattached and had not fed. A summary of the data on the fed and unfed leeches observed is given in Table 3. It will be noted that most of the leeches found on volunteers with treated clothing had not fed. Slightly more than half of the leeches on persons wearing untreated uniforms were unfed. On the other hand, most of the leeches that had attached to persons in native dress had fed. In this last group there was evidence that some leeches had completed their feeding before the end of the 30-minute exposure period and had dropped to the ground. For this reason it is believed that the actual number of leeches attacking this group was greater than indicated in the tables.

In the case of the scantily clad natives wearing impregnated socks, it was noted that the protection against leeches was excellent (see tables). Only one leech fed on a volunteer wearing treated socks. In contrast, 24 bites were noted on persons wearing untreated socks, and the barefooted members of the teams suffered 70 bites. These results were unexpected in view of the presence of large numbers of the bush-climbing leech, which normally attaches above the socks. The effectiveness may have been due to the fact that this experiment was conducted during a relatively dry period on a rainless day when the leeches were at a lower level. Little protection would be expected against leeches attached to vegetation above the knee-length socks.

It should be pointed out that the mechanical barrier of the untreated uniforms and socks was probably not as great as indicated by the data in the tables. The presence of relatively large numbers of unfed leeches on these persons was undoubtedly due to the barrier and the relatively short period of exposure. It was observed that if the leeches were given sufficient time practically all of them were able to feed.

DISCUSSION

It is apparent from the data obtained that these repellents would be of value to troops and to others whose activities exposed them to the attacks of terrestrial leeches. The effective protection of the scantily clad natives, by using impregnated socks, is of particular interest. Almost any type of undertaking in leech-infested regions must rely heavily upon the local population for porters, laborers, guides, etc. The habits of the people, the cost of outfitting them with protective clothing, and the climate, makes this method of control impractical. On the other hand, the cost of socks would be insignificant and wearing them with their sandals was readily accepted. In fact, as soon as the value of the impregnated socks became apparent in these experiments, they were prized acquisitions and were preferred to money and other items offered as wages. It should be remembered, however, that under certain conditions considerable numbers of the bush-climbing *H. picta* might be able to attack a man depending primarily on impregnated socks for protection. However, bites from the ground-inhabiting *H. zeylanica* and from *H. picta* during relatively dry periods would be prevented.

The present investigation did not include separate tests on the various components of the repellents used. M-2065 and M-2066 were as effective in our experiments as M-1960, and they had nothing in common except the emulsifying agent. This may indicate that other insect repellents will prove to be effective against leeches. However, Stammers (1946) listed benzyl benzoate among the compounds tested and reported that it had little effect against *Haemadispa*. It is also of interest that Audy (1954) showed that the insecticides aldrin, dieldrin, and lindane have little effect as toxicants against either aquatic or terrestrial leeches.

The conditions under which the present experiments were conducted gave a more stringent test of the residual effect of these repellents than is indicated by the number of washings. The volunteers were frequently exposed to heavy rains and were almost constantly walking through wet vegetation. The clothes, therefore, were saturated with water most of the time during the experiments. In effect they were "rinsed" more often than indicated.

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SUMMARY

Uniforms impregnated with insect-repelling compounds M-1960, M-2065, and M-2066, after an initial washing, afforded complete protection against the terrestrial leeches *Haemadipsa zeylanica* and *H. picta* of North Borneo. After four more washings these compounds still gave protection, although the repellency was not complete. After six washings, the repellent effects of all compounds were not sufficient to be of practical value. No noteworthy differences in repellency of the compounds were noted. Impregnated socks were found to be an effective and feasible method for protecting scantily clad indigenous people against attacks of leeches that attach to the host below the knee.

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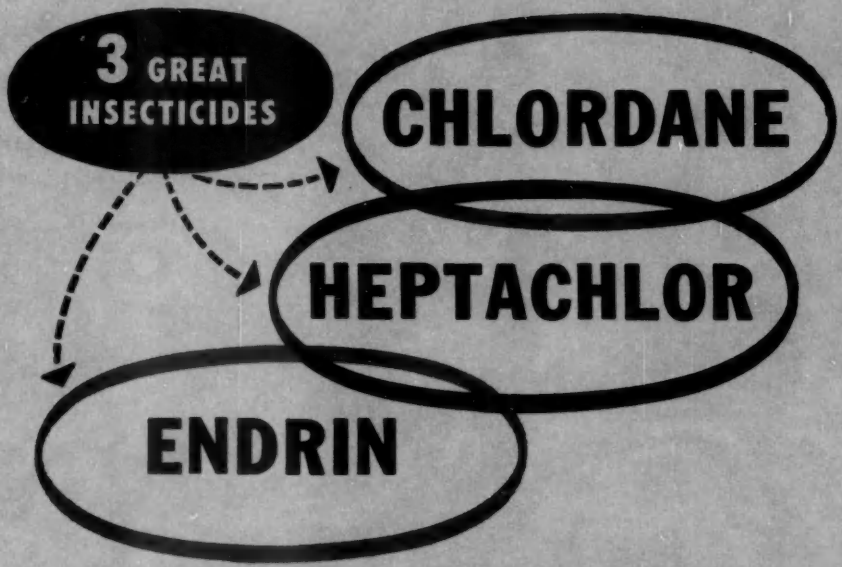

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HEPTACHLOR: Alfalfa Snout Beetle, Alfalfa Weevil, Ants, Argentine Ant, Armyworms, Black Vine Weevil, Cabbage Maggot, Clover Root Borer, Colorado Potato Beetle, Corn Rootworms, Cotton Boll Weevils, Cotton Thrips, Cowpea Curculio, Crickets, Cutworms, Egyptian Alfalfa Weevil, European Chafer, Eye Gnats, Flea Beetles, Garden Webworm, Grasshoppers, Japanese Beetle, Leaf Miners, Lygus Bugs, Mormon Cricket, Mosquitoes, Narcissus Bulb Fly, Onion Maggot, Onion Thrip, Rapid Plant Bug, Rice Leaf Miner, Salt Marsh Sand Fly, Seed Corn Maggot, Serpentine Leaf Miner, Spittle Bug, Strawberry Root Weevil, Strawberry Rootworms, Sugar Beet Root Maggot, Sweet Clover Weevil, Tarnished Plant Bug, Tuber Flea Beetle, Turnip Maggot, Western Harvester Ant, White Fringed Beetles, White Grubs (June Beetles), Wireworms . . . and many others.

ENDRIN: Budworms, Cabbage Worms, Cotton Boll Weevils, Cotton Bollworm, Cotton Fleahopper, Fall Armyworm, Grasshoppers, Hornworms, Leafworms, Rapid Plant Bug, Spiny Bollworm, Sugar Beet Webworm, Tarnished Plant Bug, Thrips.

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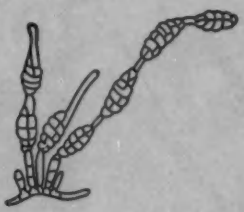
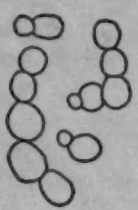


DIAGNOSTIC

. . . for the isolation, identification and cultivation of pathogenic fungi. These media are also widely used in phytopathological studies. Several are neutral in reaction, giving optimum conditions for growth of a variety of fungi. The following may be prepared as selective media by the adjustment of reaction, addition of antibiotics or other agents:



Bacto-Brain Heart Infusion Agar
Bacto-Sabouraud Dextrose Agar
Bacto-Sabouraud Maltose Agar
Bacto-Littman Oxgall Agar
Bacto-Bean Pod Agar
Bacto-Mycological Agar
Bacto-Mycological Broth
Bacto-Corn Meal Agar
Bacto-Corn Meal Agar with Dextrose
Bacto-Prune Agar
Bacto-Lima Bean Agar

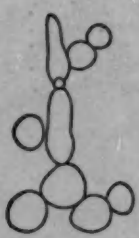
CONTROL

. . . for sanitary and sterility procedures as well as for general use in mycological procedures:

Bacto-Sabouraud Maltose Broth
Bacto-Sabouraud Liquid Medium
Bacto-Malt Extract
Bacto-Malt Agar
Bacto-Neurospora Culture Agar
Bacto-Potato Dextrose Agar
Bacto-Mildew Test Medium
Bacto-W.L. Nutrient Medium
Bacto-W.L. Differential Medium

CLASSIFICATION

. . . and nutritional studies of fungi:



Bacto-Yeast Morphology Agar
Bacto-Yeast Carbon Base
Bacto-Yeast Nitrogen Base
Bacto-Czapek Dox Broth
Bacto-Czapek Solution Agar
Bacto-Vitamin Free Yeast Base

THE DIFCO MANUAL, *NINTH EDITION,*
including descriptions of these media and their use,
is available on request.

DIFCO LABORATORIES

DETROIT 1, MICHIGAN